

Journal of  
Pathology and Bacteriology

# The Journal of Pathology and Bacteriology

The Official Journal of  
the Pathological Society of  
Great Britain and Ireland

EDITED BY

M J STEWART

H D WRIGHT G R CAMERON



*FOUNDED IN 1892 BY GERMAN SIMS WOODHEAD*

VOLUME FORTY SIX

Oliver and Boyd

London 98 Great Russell Street, W C

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## THE RUGOSE VARIANT OF VIBRIOS

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(PLATE I)

AMONG the variant colonial forms noted by Balteanu (1926) in his study of cholera and El Tor vibrios was one to which, from its corrugated growth-habit on agar, he gave the name "rugose variant," a form presumably corresponding to Baerthlein's (1912, 1918) variant II. From a detailed description of the rugose variant Balteanu was probably deflected by the fact that it proved unstable in culture. His attention was occupied with a more stable "opaque" variant which is perhaps a cultural form of much the same *genre*.

During recent years the rugose variant has been indicated as the extreme rough form of vibrio culture and a considerable literature has grown up round this interpretation (*eg* Linton, Shrivastava and Mitra, 1934-35, Linton, Mitra and Mullick, 1935-36, Linton, Mitra and Seal, 1936-37, Seal, 1936-37). From such an interpretation, and from various conclusions based on it, I have briefly demurred (White, 1935, 1937) but it is desirable that the nature of the frequently encountered rugose culture should receive fuller consideration.

Of all the colonial types met with in vibrio cultures on agar, the rugose variant most invites photography, but though it has been often portrayed, published photographs hardly do justice to its fully developed peculiarity. It is hoped that figs 1c and 1d, representing rugose derivative colonies of *V. cholerae* strain Rangoon S, remedy this defect and obviate the necessity for elaborate description. Typical smooth and rough colonies of the same strain, to which, on account of the part it has played in

Dr R W Linton's studies, I have given special attention, are represented in figs 1a and 1b respectively, for comparison.

Another culture studied with particular care was the problematical rugose strain "Rangoon rough 2," the S or R nature of which may be accepted as the specific issue. During the latter part of the research, single cell cultures of Rangoon S, of one of its rugose derivatives, and of Rangoon rough 2 have been received from Dr Linton and have been valuable in checking up observations.

A comparison of relevant cultural features of typical smooth, rough and rugose cultures applicable to *V. cholerae* and most other vibrios grown on agar is given in the accompanying table.

The rugose type of colony is apt to appear at any time in platings of vibrio cultures, particularly perhaps in platings of ageing peptone water cultures, and it is possible that peptone concentrations of the order of 5-10 per cent favour its development. Such colonies often abound in platings of vibrios surviving specific bactericidal tests, a fact which suggests that the variants resist, or are protected against, activated immune serum. Both S and R cultures may assume this habit of growth.

While the general character of selected rugose races is readily maintained by selection, there is perpetual reversion to the normal growth habit. Sooner or later during incubation of a plate culture reverting outgrowths develop at the margins of the rugose colonies, the degree of reversion varying from culture to culture. The instability of the rugose variant, contrasted with the stability of the ordinary R form, is in itself sufficient to render it improbable that rugosity is the culmination of roughness. Rugose derivatives of S cultures revert directly to the typical S form, rugose derivatives of R cultures directly to the usual R form—another significant point.

It requires no great intuition to guess that the coherence of the rugose growth is not due to a special interlacing of cells as Scal (1936-37) has suggested, but to the fact that the cells are embedded in a glutinous intercellular substance or capsular material.

In the microscopic demonstration of this condition I have found helpful smears made without the use of water, spread by the sweep of a glass slide, flame-fixed and stained after the manner of Hiss with gentian violet followed by copper sulphate solution.

The early cultures (6-8 hours) of typical S *V. cholerae* and related vibrios so treated show closely ranged vibrios of classic form lying in general parallel to the stroke of the slide, and a meagre faintly staining background of intercellular material: here and there may be found a deeply staining granule or spherical element. With further incubation the character of the culture as revealed by stained smears and moist preparations, changes: spherical forms, budded off terminally and laterally from the vibrios in vast numbers.

TABLE

	Normal S culture	Typical R culture	Rugose culture (derived from S culture)
Agar surface colony 1 Appearance	Circular, moderately raised glistering and moist looking finely granular under the lens and of variable transparency or turbidity. Occasionally essentially S colonies may show the appearances usually associated with roughness.	As a rule differs but slightly in general appearance from the S colony and cannot with certainty be recognised by simple inspection usually duller of surface and more coarsely granular and may show irregularity of outline and flattening, transparency may be increased or diminished. The out- ward peculiarity of the colony affords no measure of the intensity or persistence of the roughness of the culture (Of Shousho, 1923 24 Gayle and Sen Gupta, 1932 33 Dambovicanu, Combesco, Wisner and Soru, 1934)	The 18 hours colony is small, much raised and refractile. It increases more rapidly in diameter with further incubation and develops the superficial corrugation, irregular or radial or both, which is character- istic. Opaque yellowish or yellow in colour, opaque and tint deepening with age. In older cultures a vitreous or granular "corona" may be exuded from the margin of the colony
2 Consistence and adherence	Semi fluid never adherent to the medium	Dry brittle never adherent to the medium	Tough or gelatinous, adherent to the agar site of displaced colony marked by turbidity
Dispersibility and agglu- tination of agar growth in NaCl solution	Growth disperses readily with perhaps some initial firmness. Dense suspensions in 1.7 per cent salt solution may show some precipitation of slime trapping a few vibrios	Growth disperses readily in saline (0.85 or 1.7 per cent NaCl) with initial firmness, but quickly and completely agglutinates so that the clumps float in a lumped fluid	Growth disperses only partially and with difficulty in salt solution Vibrios once dispersed are insensitive to NaCl



notwithstanding the negative observations of Seal (1935), sooner or later and increasingly become the dominant elements. It is unnecessary here to discuss the origin and nature of the spherical buds in detail. pending effective reinvestigation, I reject, as does Henrici (1925), the view of Lohnis (1922) and others that they are special reproductive cells (conidia, gonidia), on the other hand I am disinclined to regard them all as involution forms, pure and simple, though many are doubtless pathological. there is on the whole some evidence to support the suggestion of Stamm (1914) that some of these spherical buds have a particular secretory function, tending to protect the culture against desiccation.

With the appearance of numerous spherical forms, the intercellular material of the ordinary S culture increases somewhat in quantity and in depth of staining, but though it may confer on the growth a certain sliminess in salt solution it, of course, remains freely "soluble" and does not prevent even dispersal of the cells.

In the R culture appearances are very similar to those in the normal S culture save that in smears of the type referred to the vibrios tend to remain densely clustered and to be disposed without reference to the stroke—doubtless an expression of the dryness of the intercellular deposits, which as in the ordinary S culture are small in amount, show no local accumulations and do not appreciably hinder dispersal of the culture in salt solution.

Setting aside the somewhat peculiar culture, Rangoon rough 2, for later consideration, it may be said that smears of the rugose culture convey the impression that there is a wider spacing of the units than in ordinary S and R cultures, and here and there, even in the young culture before spherical bodies appear in appreciable numbers are to be found structureless masses of intercellular material often taking the Hiss staining with moderate intensity. As the culture develops the masses of accumulated secretion seen in smears become larger and more numerous and are usually found to contain or to be associated with clusters of deeply staining spherical forms or vibrios. A small centre of secretion of this type—a colony within the colony—is shown in fig. 2.

When the rugose culture is ground in saline the vibrios are in variable part dispersed, if the temperature is raised to 55° C the proportionate dispersal is increased. The undispersed residue collected by gentle centrifugation forms a mucinous mass. Smears of this material, fixed and stained, show cellular foci and sparsely distributed vibrios embedded in faintly staining secretion.

Despite the development of large amounts of gelatinous or mucoid intercellular material, in all the rugose cultures examined I have been unable to demonstrate with certainty in any save Rangoon rough 2 individual capsulation of the cells: the vibrios seemed to be set in a common zoogloea.

## RUGOSE VIBRIOS

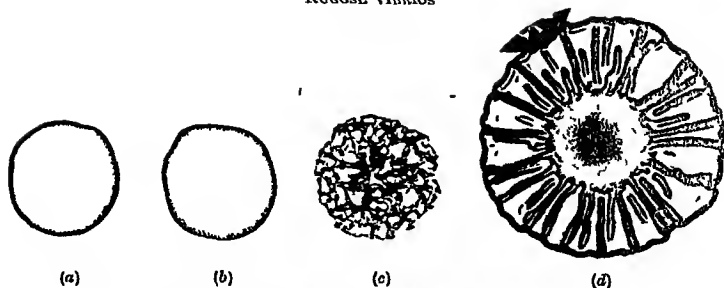


FIG 1—*V. cholerae* strain Rangoon smooth Agar surface colonies, incubated at 37° C  $\times 9$

- (a) Typical smooth colony (18 hours growth)  
 (b) Typical rough colony (18 hours growth)  
 (c) Rugose colony with irregular corrugation (3 days growth)  
 (d) Rugose colony with radial corrugation (3 days growth)

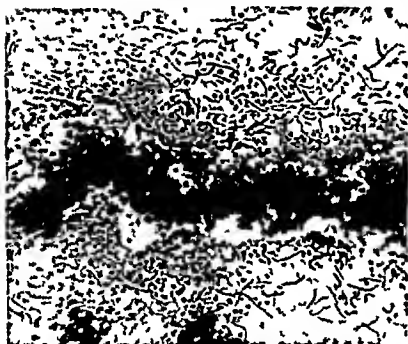


FIG 2—*V. cholerae* strain Rangoon smooth Smear of rugose growth from agar (36 hours growth at 37° C stain Hiss,  $\times 1250$ ) showing cluster of spherical forms embedded in their faintly staining gelatinous secretion

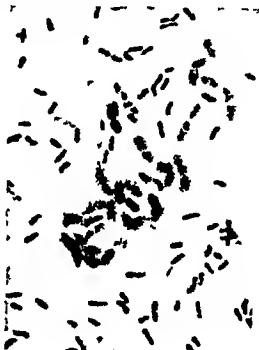


FIG 3—*Vibrio* Rangoon rough 2 Smear from peptone water culture pellicle (48 hours growth at 37° C stain Hiss,  $\times 1250$ ) showing group of capsulate forms



FIG 4—*Vibrio* Rangoon rough 2 Smear from same pellicle as in fig 3 similarly stained and enlarged showing deeply staining oval bodies  $\times 1250$



FIG 5—*Vibrio* Rangoon rough 2 Smear from same pellicle as in figs 3 and 4 stained with nigrosin to show the structure of the oval bodies  $\times 1250$



The culture Rangoon rough 2 differed from the other rugose races studied in being subject, after several days' incubation at 37° C or on the bench, to a rapidly extending slimy degeneration of the intercellular substance rendering the cells readily and permanently dispersible in saline, in the plump bacillary form of its units and in failing to generate spherical buds. In smears of the agar growth stained in various ways it could often be determined that the gelatinous nodules which it contained were composed of capsulate forms with somewhat diffuse and confluent envelopes or of closely packed rather deeply staining ovoid bodies which could sometimes be seen to contain a number of cells arranged in different directions. Since more satisfactory preparations could be obtained from the gelatinous pellicle of peptone water cultures, these have been employed for purposes of illustration. Smears of the pellicle, fixed and Hiss-stained, offered a microscopic picture of remarkable variety: scattered short bacilli showing no evidence of capsulation, groups, cords and webs of patently capsulate forms (fig. 3) and clusters of large deeply staining oval bodies (fig. 4) in which it was often difficult to make out any definite structure and usually surrounded by a diffuse capsular haze. Examined by dark ground illumination, moist preparations appeared to be entirely composed of short bacilli: capsules and large ovals were invisible. At the suggestion of Professor A. Fleming, I made a trial of "negative" staining by nigrosin, both by mixture of the stain with the growth before smearing and by applying it to dried smears: thereupon the nature of the oval bodies was disclosed. The stain, apparently penetrating to some extent the substance of these bodies, left sharply defined the unstained bacteria within, exposing them as magnificently capsulate forms (fig. 5).

On the whole it seems probable that this apparent difference as regards capsulation of Rangoon rough 2 from the other rugose races studied is a matter of degree or case of demonstration. The essential point is that the rugose growth habit in vibrios results from abnormally active secretion of mucinous material while the character of the rough culture is due, as I have shown (White, 1934, 1935, 1936), to failure to secrete or form the specific polysaccharide. It is difficult to imagine two forms of change more opposite in nature.

It seems possible that the corrugation of the rugose culture is due at least in part to variations in secretory activity in different regions of the growth, the effect of which may be accentuated by the hygroscopic properties of the secretion. The case with which change occurs to and from the rugose condition perhaps suggests that the rugose character is due to an intensification of the normal secretory processes rather than to a special type of activity.

Whether the intercellular or capsular material of the rugose

vibrio has any special serological property I am not yet certain, but it may readily be shown that the organisms which, by grinding in saline, can be liberated from a rugose culture of S origin, are after washing normally agglutinable by the corresponding smooth O antiserum. Emulsified particles of the mucoid residue from grinding are but slowly agglutinable with smooth serum and appear to have an inhibitory effect on S agglutination.

### Conclusions

The rugose growth-habit in vibrios has no connection with roughness, it may be assumed by S and R races alike. When they revert to the more usual growth-habit of vibrios, rugose races return to the S or R condition from which they were derived.

The rugose condition is due to secretion by the culture of a gelatinous intercellular substance or definite capsules.

My particular thanks are due to Mr F. Welch who, by the courtesy of Mr J. E. Barnard, F.R.S., has prepared the microphotographs here reproduced, sparing no pains to achieve the best possible presentation of the material.

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# THE CANINE PROSTATE IN RELATION TO NORMAL AND ABNORMAL TESTICULAR CHANGES

S ZUCKERMAN and T McKEOWN

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In a previous study (Zuckerman and Groome, 1937) it was reported that 8 of 9 canine prostates collected at random were larger than the size usually regarded as normal. As very little appears to be known about the normal range of variation, the high incidence of enlargement in this sample of 9 specimens made it interesting to enquire whether or not the canine prostate enlarges gradually throughout life. It proved impossible to obtain material from official R S P C A stations or "dogs' homes" for such a study. As a rule, however, dogs killed at these places are disposed of for certain purposes of trade, and through the courtesy of Messrs Harrison Barber Ltd, slaughterers, we succeeded in collecting the testes and prostates of 243 animals.

## *Material and methods*

Carcases are brought to the slaughterer's yard at periods varying from a few hours to two days after death. The prostates and testes were immediately removed and, after the prostates had been measured, were placed in 10 per cent formol. The breed and approximate age of each animal were diagnosed by Mr F J Drew, an employee of Messrs Harrison Barber whose entire time for over 20 years has been spent in handling dogs.

After 10 days' fixation, the volumes of the prostates were determined by water displacement in a measuring cylinder. Transverse sections through the centre of each organ were prepared and stained with hematoxylin and eosin. Sections of each testis were also prepared. Post mortem changes were in general more marked in the prostates than in the testes, most of which proved to be in remarkably good condition.

The first 9 of our series of canine prostates have already been described (Zuckerman and Groome). The 10th, 11th, 12th and 213th specimens were obtained from veterinary surgeons\*. Nos 13-149 of the remainder were obtained at Messrs Harrison Barber's and were collected at random from the available dogs without selection of breeds. Nos 150-212 were taken only from fox terriers, Manchester terriers and spaniels. Nos 214 to 243 were taken from any dogs whose testes were either abnormal or absent.

---

\* We wish to express our thanks to Dr E G White of the Royal Veterinary College, London, and to Mr Charles Roberts, M R C V S, of Tunbridge Wells for their kindness in sending us these specimens.

TABLE I.  
*The size of the canine prostate in relation to breed, age and body-weight.*

Breed	Sexually immature				Mature (up to 5 years).				Mature (6 years and over)				Totals
	No of specimens	Mean body-weight (kg)	Mean diameter of prostate (mm)	Mean volume of prostate (c.c.)	No of specimens	Mean body-weight (kg)	Mean diameter of prostate (mm)	Mean volume of prostate (c.c.)	No of specimens	Mean body-weight (kg)	Mean diameter of prostate (mm)	Mean volume of prostate (c.c.)	
Box-terrier	3	6.7	11.0	1.2	24	8.8	20.4	7.0	24	8.6	24.0	9.5	51
Manchester terrier	1	6.8	9.0	1.0	14	8.0	17.0	3.7	8	9.5	27.8	15.6	23
Wire-haired terrier	2	8.2	10.5	1.3	1	6.8	16.0	4.0	0				3
Scottish terrier	0	..			4	8.3	24.0	9.7	1	9.1	19.0	5.0	5
Irish terrier	0				2	11.1	16.5	5.0	2	9.1	25.0	10.5	4
Yorkshire terrier	0				0				1	10.0	26.0	8.0	1
Spaniel	3	11.8	15.0	2.6	9	11.0	22.0	7.6	11	12.1	30.7	18.1	23
Collie	1	16.0	11.0	2.0	3	14.2	21.3	8.7	2	20.5	36.0	26.0	6
Mentian	0				2	14.1	21.5	8.5	3	22.7	31.0	25.3	5
Retriever	1	8.6	12.0	3.0	1	18.6	15.0	4.0	2	19.1	40.0	30.6	4
Airedale	0				1	16.3	11.0	2.5	3	19.5	39.3	35.3	4
Greyhound	0				2	17.3	18.0	5.0	3	22.1	30.0	13.0	5
Pomeranian	0				1				2	5.7	21.0	7.5	2
French pug	0				0	6.8	24.0	11.0	0				1
Mastiff	0		..		1				0				1
Chow	0				0				1	36.4	42.0	33.0	1
Pekinese	0				0				1	20.9	31.0	20.0	1
Setter	1	6.3	9.0	1.5	0				1	4.5	16.0	5.0	1
Dalmatian	0				0				0				1
Box-hound	0				1	17.7	33.0	13.0	1	15.9	30.0	21.0	1
Mongrels	4	7.0	10.5	1.1	30	8.9	19.8	6.3	23	11.6	25.4	12.6	57
All dogs	16	8.6	11.3	1.63	95	9.6	19.9	6.46	89	12.1	27.3	14.7	200

*The size of the canine prostate*

The observations on the size of the prostate which are summarised in table I relate to the 200 specimens 13-212 inclusive. The diameter of the organ given is the mean of the maximum ventro-dorsal, crano-caudal and transverse dimensions. Sexual immaturity was determined by the absence of spermatogenesis and by an infantile condition of the seminiferous tubules. Dogs become sexually mature when about one year old and it is of interest that there were only four animals which Mr Drew estimated to be over a year and which did not satisfy the histological criteria of maturity. No histological check for the estimates of age of the mature dogs is available, but the mean size of the prostate in the presumed oldest group of animals is almost invariably larger than in the group which includes dogs of only up to 5 years of age.

TABLE II

*Partial correlation coefficients for body-weight and age of dogs  
in relation to prostate volume*

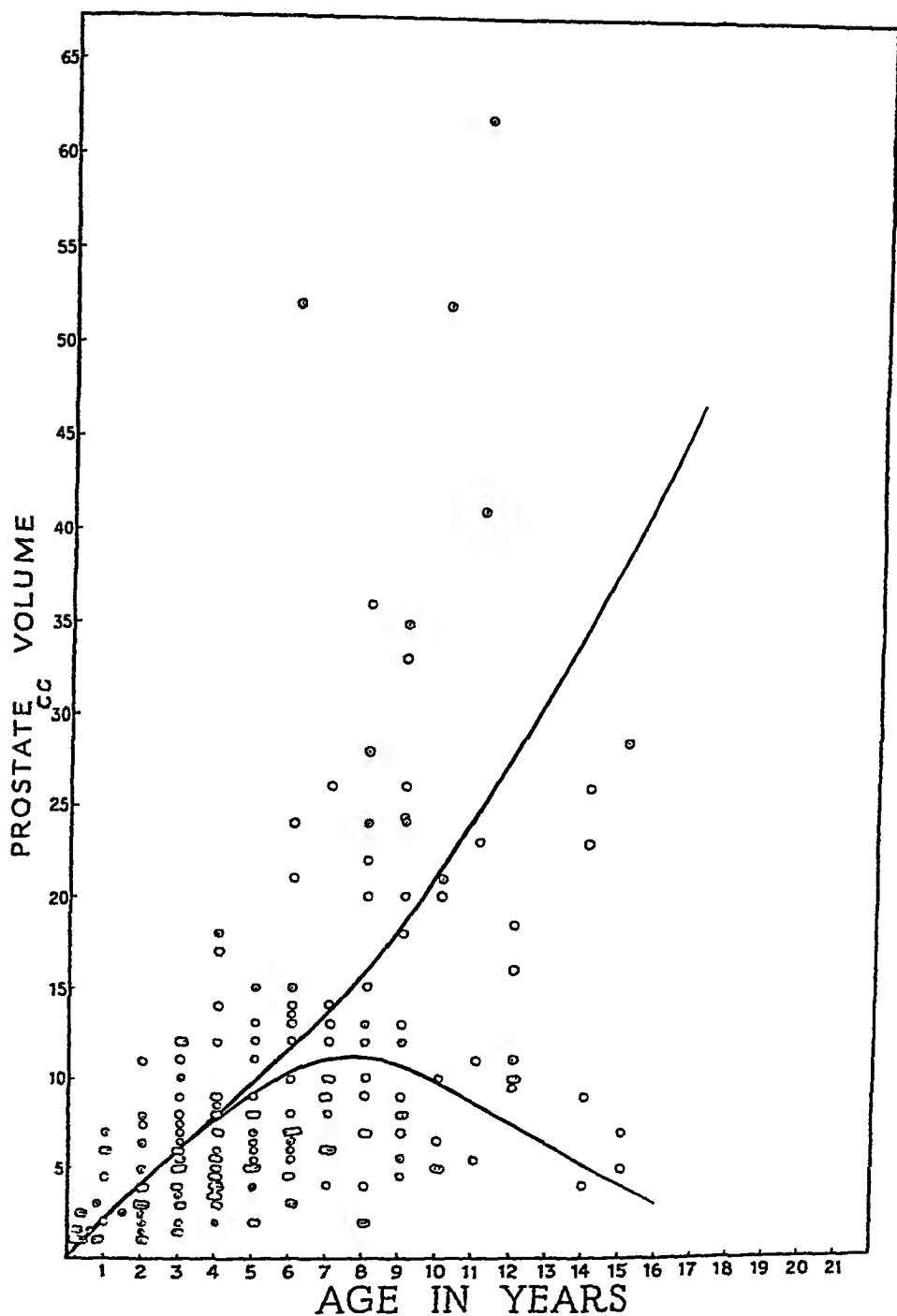
	<i>N</i>	<i>R ap w</i>	<i>R wp a</i>
Fox terriers	47	0.3040	0.2412
Spaniels	20	0.4708	0.5592
Manchester terriers	20	0.6379	0.1690
All dogs	200	0.3215	0.4547

*R ap w* is the coefficient of correlation between age and prostate volume, the effect of body weight being eliminated. *R wp a* is the coefficient of correlation between body weight and prostate volume the effect of age being eliminated. The coefficients in black type are significant.

The available data do not disclose any orderly relation of body-weight and prostate-size between the different breeds. Thus the prostate in a breed such as the French pug may be larger than in alsatians or collies of corresponding age-groups. Attempts to analyse the data by means of partial correlation coefficients for age and body-weight were not entirely successful. The coefficients obtained are collected in table II and it will be seen that all four possible results of the correlation analysis have been obtained in the four groups studied. Where all the specimens are considered together, both age and body weight appear to influence the size of the prostate.

It is clear from tables I and II that the prostate may grow considerably after sexual maturity is reached and that in each breed growth may continue into late life. This is also demonstrated by the figure, which is a scatter diagram of the age-prostate-volume relation in the 200 specimens 13-212 inclusive (all breeds). The great variance of the data in each year of life as shown in this figure





The size of the canine prostate in relation to age

provides the reason for the relative failure of statistical analysis \* Similar variance is revealed in scatter diagrams constructed for animals of a single breed (e.g. 51 fox-terriers, 23 Manchester terriers, 23 spaniels), and in scatter diagrams illustrating body-weight-prostate-size relations Although the largest prostates are usually found in the older animals, relatively small prostates also occur in old dogs The lines drawn in the figure illustrate two extreme trends of change in the prostate with age The first leads to progressive enlargement, the second to involution Thus interpretation of the data is supported by the histological evidence

The data summarised in table I confirm the view stated previously (Zuckerman and Groome) that 8 of the first 9 prostates in our series were "enlarged"

### *The histology of the canine testis and prostate*

The prostate proved to be the same anatomically and histologically in all the breeds investigated The following observations refer to the whole of the 243 specimens examined (table III)

Histological study of the testes showed that 19 of the dogs were sexually immature The prostates of these animals were infantile in type the glandular acini were not expanded, the epithelium was neither high nor functional and the ratio of epithelial to fibro-muscular tissue was low The remaining 224 animals were mature In 177 spermatogenesis was in progress and the testes were normal Lymphocytic infiltration of the testes was, however, occasionally to be seen and a few testes were abnormally fibrotic In some specimens a few seminiferous tubules appeared as solid rods of cells Spermatogenesis was in abeyance in three animals which were estimated to be more than 10 years old (the testes nevertheless maintaining their normal histological form), and in four younger animals which presumably may have suffered from some nutritional deficiency † The amount of interstitial tissue varied, but post-mortem changes made it impossible to assess individual differences in this character with any accuracy All stages intermediate between a somewhat immature condition of the prostate on the one hand and glandular hyperplasia or glandular involution on the other were observed in the 177 animals in whom spermatogenesis was proceeding

The general arrangement of the lobules in the canine prostate has been described previously (Zuckerman and Groome, figs 4, 5 and 7) In the fully mature normal prostate the lobular arrangement

\* It is a curious coincidence that in our data there proved to be a fairly close relation between the mean body weight in pounds of each age group of dogs and the mean diameter of the prostate in millimetres for the corresponding groups

† The male dog does not experience a reproductive season, and in normal health spermatogenesis proceeds continuously throughout the year

remains distinct and the glandular acini, which are not greatly distended, are lined by tall, active cylindrical epithelium\*. The vascularity of the normal prostate varies considerably and considerable hæmorrhage was present in one specimen (no. 12). Inflammatory lesions are not uncommon and prostatoliths are sometimes visible to the naked eye.

Signs of involution are present in several prostates. The process has affected the greater part of the organ in a few of the older animals, especially those in which the prostate is small. In such cases the ratio of fibro-muscular tissue to epithelial elements is much increased, the glandular cells are low and the acini are reduced in size. It is of interest that signs of prostatic involution may be present even when spermatogenesis is occurring.

Various degrees of glandular hyperplasia are also displayed in the present series of specimens, but none much greater than that depicted in fig 15 of our previous study. The more excessive the condition the less does the prostate maintain its orderly lobular arrangement and the greater are the number of papillomatous extensions into the distended acini. The process is best marked in the largest prostates in our series. Isolated adenomatous nodules such as are seen in the human prostate, of the sort that can be "shelled out," were not seen in any of our specimens.

Involution of the prostate was observed in only one of the 7 mature animals in whom spermatogenesis had stopped; the prostates of the other 6 were normal. These 7 animals, as noted above, were presumably either senile or suffering from some nutritional deficiency.

The 5 prostates listed below were obtained from mature animals in whom no scrotal testes were found and who at autopsy were therefore presumed to be castrates.

No	Estimated age (years)	Volume of prostate (c c)
57	9	18
67	3	12
164	6	24
220	6	2
217	10	6

The prostates of nos 57, 67 and 164 show, however, few signs of involution, and it is therefore possible that these animals were cryptorchids. Those of nos 220 and 217 were in an advanced condition of glandular involution, a finding which suggests that

\* The material now available makes it clear that the epithelium of the normal prostate figured in the paper referred to above (fig 7, Zuckerman and Groome) was not fully functional, even though the specimen was recovered from an animal whose testes were fully mature.

these animals had in fact been castrated. The prostate of 217 was an almost purely fibro-muscular organ.

*Changes in the prostate other than simple hyperplasia  
or involution*

Only 7 of the 243 prostates in our complete series show changes other than those described above. Carcinoma is present in the first (no 225), which is otherwise involuted, and the second (no 213) shows a papilliferous cystadenomatous change accompanied by considerable hyperplasia of the smooth muscle. The remaining 5 anomalous specimens (nos 1, 11, 222, 227 and 242) have undergone the metaplastic process described under the heading "dog 1" in our previous paper. This process converts the cylindrical glandular cells into a rapidly growing stratified squamous epithelium, with the result that the glandular acini are transformed into cysts of varying size, filled with desquamated epithelium. Since this process can be reproduced experimentally by the injection of oestrogenic substances, its spontaneous occurrence may be attributed to similar stimulation occurring naturally. Dogs 13-212, as noted above, were examined at random without reference to abnormalities of the testes. None of them shows squamous metaplasia of the prostatic epithelium. It is unfortunate that the incidence of this latter condition cannot be estimated on the available material.

*The prostate in relation to testicular abnormalities*

Neoplastic changes in the testes had occurred in the 7 anomalous dogs referred to above\*. Testicular tumours were also present in 28 other dogs in our series and in these the prostates were either normal or in a state of simple glandular hyperplasia or glandular involution (tables III and IV). Only 6 of the total of 35 testicular tumours were found in the series of 200 dogs which were collected at random without any reference to abnormalities of the testes. Twenty-eight were found in animals whose testes were larger than normal. The 29th tumour was found in dog 1 (see footnote).

The neoplastic testes varied in appearance. Some were solid and homogeneous, others nodular. Many showed regions of hæmorrhage and a few appeared to be mostly converted into cysts containing gelatinous material.

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\* In our previous paper it was stated that the testes of dog 1 were small and that histological examination, which was somewhat limited owing to imperfect fixation, showed that spermatogenesis was in abeyance or much slowed down at the time of the animal's death. This statement was based on the examination of two blocks of tissue. The testes have since been sectioned serially (in view of the condition of the testes in other dogs in our series whose prostates had undergone squamous metaplasia) and an encapsulated spherical tumour 6 mm in diameter was found towards the lower pole of one testis. This tumour has been diagnosed histologically as an adenocarcinoma (see above).

The histological classification of testicular tumours presents considerable difficulty and there is much difference of opinion between authorities on the subject. The canine testicular tumours of which we have found a record are (a) one seminoma reported by Imamaki (1931), (b) two interstitial growths recorded by Ball (1922) and (c) three tumours of cryptorchid testes reported by Gieulich and Burford (1936)—not specifically diagnosed but stated to be either seminomas, embryomas or adenocarcinomas, together with (d) a few observations by Peyron (1922) on the part played by Sertoli cells in canine testicular tumours. Consequently we have classified our own series on the basis of human pathology.

TABLE III

*Testes and prostates of 243 dogs of different breeds and age.*

Testes								Prostate							
Immature	Mature							Immature	Mature						
	Normal	Scrotic	? Nodular of ducts	Cystic	? Cryptorchid	Seminoma	Adenocarcinoma		Intersititial tumour	Normal, including glandular hyperplasia and involution	Full involution	Squamous metaplasia	Carcinoma		
19	177	3	4	2	3	17	15	1	19	177	1	2	2	5	1
										2					
										1	2				
										3	2				
										15	2				
										5	1	5			
										3					

Three of our canine tumours are interstitial in origin, the remaining 32 being derived from the cells of the seminiferous tubules. The interstitial tumours resemble the corresponding human new-growths (cf Oberndorfer 1931) and appear to be identical with the two canine interstitial tumours described by Ball. They are composed of large eosinophilic cells with relatively small nuclei and somewhat vacuolated cytoplasm. The cells are generally arranged in perivascular strands and the growths are very vascular and occasionally haemorrhagic. The swollen interstitial cells are almost identical in form with those seen in the testes of intersexual pigs and they also resemble both luteal cells and the cells of the adrenal cortex.

The 32 tumours of germinal origin have for convenience been divided into the two groups, seminoma and adenocarcinoma.



teratoid or mixed tumors") are true embryomas Bang *et al.* (1935) and Hamburger *et al.* (1936), on the other hand, regard seminomas and mixed epitheliomas as being respectively the non-differentiated and differentiated forms of tumours arising from the cells of the seminiferous tubules. They have been successful in correlating their histological findings with the results of endocrine analysis of the urine and of radiological treatment of the patients whom they investigated. Their further view that the polycystic teratomas and the mixed epitheliomas have a different origin is also supported by the results of endocrine analyses

The canine seminoma, of which there are 17 in our series, is in general the same as the corresponding human tumour. The growth consists of a fairly solid cellular mass with a relatively fine stroma which is variably infiltrated with lymphocytes. The cells are irregular in size and in general their nuclei are relatively large. Multinucleated and dividing cells are common. The degree of vascularity, hæmorrhage and necrosis varies from specimen to specimen, as does also the number of recognisable seminiferous tubules.

The remaining 15 tumours have been classified as adenocarcinoma and they correspond to the mixed epithelioma of the human testis. Essentially they represent a malignant development of all the cells of the seminiferous tubules, including the cells of Sertoli (which do not take part in the formation of seminomas). The relative parts played by the cells of Sertoli and the germinal cells in the production of the tumour vary. The canine adenocarcinoma differs from the mixed epithelioma of the human testis in being less differentiated and polymorphic and in the obvious participation of Sertoli cells in the growth.

In a few cases the tumours are isolated in the testis, which may be small in size. When the cancer is further developed, the testes may be entirely disorganised and in section appear as cystic organs containing blood and coagulated gelatinous material. The extent to which sperm tubules survive in normal form and the degree of their compression vary.

Histological examination shows that the general form of the seminiferous tubules is often maintained in this class of tumour, but tubules are sometimes greatly expanded and much altered in shape. Usually, too, there is a considerable fibrous stroma. The degree of proliferation of the germinal epithelium varies and both typical seminoma cells and elongated Sertoli cells with relatively small nuclei may be recognised. The disposition of the cells frequently suggests that they have been separated from each other by some fluid secretion. Occasionally, too, the tumour presents a syncytial arrangement of what appear to be mainly Sertoli cells. The important part played by these cells in canine

testicular tumours has been emphasised by Poyron Necrosis and hæmorrhage are frequent and sometimes considerable \*

One of the specimens falling into this class of tumour was obtained from a fox-terrier (no 11) which was autopsied because its scrotum had become enlarged and inflamed. During the last six months of its life the skin of its trunk had also become irregularly pigmented in black patches. Its penile sheath and nipples had also become much more prominent. There was a large hydrocele of the right testis, which was swollen. A small nodular growth projected from its postero lateral surface. The left testis was small. The prostate was enormously enlarged and histological section showed that the lining of it had undergone squamous metaplasia. Necrotic metastases of the testicular growth were found in both liver and spleen. The medullary cells of the adrenals appeared to have undergone hyperplasia.

The correlation of the condition of the prostate with the testicular tumours raises several matters of interest (table IV). Thus all but 2 of the 17 dogs with seminomas had either normal or hyperplastic prostates, the prostates in the 2 exceptional animals being involuted. This finding suggests that the secretion of gonadal hormones was normal in these animals (normal in the sense that the prostates would present the same appearance in a sample of similar size picked at random from dogs whose testes were not affected by gross pathological change). It is also in harmony with the results of the endocrine analyses of Hamburger *et al* (1937), which indicate a "rather normal testis function" in patients suffering from seminoma, and with Parkes' finding (1937, personal communication) that seminomas in capons produce neither male nor female hormone. (Seminomas, it may be noted, occasionally develop in the scar which replaces the hilum of the removed gonads of cocks.)

The prostates of the 3 animals with interstitial testicular tumours are also of interest in so far as they suggest that the tumours were not producing an excess of androgenic hormone. This finding, again, is in keeping with the fact that the testes of intersexed pigs do not produce an excess of male hormone—as indicated by the condition of their accessory reproductive organs—in spite of the relative increase of their interstitial elements (Baker, 1924-25).

On the other hand the condition of the prostate in the 15 dogs suffering from adenocarcinoma of the testis shows that a considerable change had occurred in the sex hormone metabolism of these animals. In no prostate was the glandular development sufficiently

\* The brief description given by Groulich and Burford of the tumours they found in the cryptorchid testes of three dogs suggests that the growths were of the kind that we have described as "adenocarcinoma." This view is supported by the fact that squamous metaplasia had occurred in two of the three animals they studied, as it had in five of our series. Imamura's specimen also appears to have been an "adenocarcinoma," and not the type of tumour to which we have referred as "seminoma."



pronounced to merit the description "hyperplasia," a fact suggesting that there was no excess of male hormone in any of the animals concerned. In 4 prostates the glandular system was greatly involuted and in 2 of these the whole organ had become much reduced in size. In 5 others the glandular system had undergone squamous metaplasia, a finding which indicates oestrogenic stimulation. Presumably the oestrogenic hormone had been elaborated by the tumour tissue. In view of the fact that Sertoli cells had played a part in the building up of these tumours, it is noteworthy that these cells are homologous with the granulosa cells of the ovarian follicles (Witschi 1932), and that the latter were for long considered to be the primary source of oestrogenic hormone. Furthermore it is of interest that human patients suffering from mixed epitheliomas of the testis have possessed feminine characters (Menetrier *et al*, 1922) and that 6 patients suffering from mixed epitheliomas of the testis who were investigated by Hamburger *et al* (1937) excreted more oestrogenic hormone than normal.

The occurrence of carcinoma of the prostate in one of the 15 animals which had an adenocarcinoma of the testis is probably a fortuitous circumstance. In view, however, of the fact that this type of testicular tumour produces oestrogenic hormone, it is necessary to note that carcinogenic and oestrogenic substances often overlap in function (Dodds, 1935).

### Summary.

1. Examination of 243 dogs of all ages and breeds shows that the size of the prostate is very variable in each age group. After about the eighth year of life, the prostate may either undergo involution or continue to enlarge, the two changes being equally common. Where all the specimens are considered together by the method of partial correlation coefficients, both age and body-weight appear to influence the size of the organ. Similar analysis of 47 prostates of fox-terriers failed to show any correlation with either age or body-weight.

2. Enlargement is due either to glandular hyperplasia or, more rarely, to squamous metaplasia of the cylindrical prostatic epithelium which results from spontaneous oestrogenic stimulation. Involution is shown by the regression of the glandular acini and by a reduction in size of the whole organ. The most pronounced involution was observed in two castrated dogs.

3. Testicular tumours were present in 15 dogs. Three were composed of the interstitial cells and the prostates of these animals were normal, one being involuted. Seventeen were seminomas and 14 corresponding prostates were also normal, 2 being involuted. The remaining 15 have been classified under the term "adeno-

carcinoma" and are homologous with the mixed epithelial testicular tumours of human pathology, from which they differ in being less differentiated and polymorphic. Nine of the corresponding prostates are normal, 4 being involuted. Carcinoma had occurred in the tenth prostate and the remaining 5 had undergone squamous metaplasia, presumably as a result of stimulation by oestrogenic hormone elaborated by the tumours. The oestrogenic potency of these adenocarcinomas is related to the fact that Sertoli cells take part in their formation and that these cells are homologous with the granulosa cells of the ovarian follicles.

Our thanks are due to Professor M. J. Stewart, who in the first place suggested this investigation. We are also deeply grateful to Drs J. A. Murray, F.R.S., C. Hamburger, F. Bang and J. Nielsen for their help in the diagnosis and classification of the testicular tumours and to Dr R. B. Fisher for assistance in the statistical analysis of the data. We also once more desire to record our appreciation of the care with which Mr R. Munro prepared the sections.

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# THE INCIDENCE OF THE VARIOUS SEROLOGICAL TYPES OF *STR AGALACTIÆ* IN HERDS OF COWS IN GREAT BRITAIN

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In a recent publication (Stableforth, 1937) it was shown by agglutinin absorption tests that five serological divisions exist among British strains of *Str agalactiæ* and that these fall into three main types, 1, 2 and 3, of which the sub-types are called 1a, 1b, 2a, 3a and 3b respectively. They have been arranged in main and sub-types because, while quite distinct by agglutinin absorption tests, they show certain marked cross reactions in direct agglutination and precipitation tests. Most British strains fall into one or other of these sub-types. Types now called 1a, 2a and 3a were originally called types a, b and c (Stableforth, 1932), but were redesignated for reasons given in the later paper (1937).

The purpose of the present report is to give certain facts regarding the distribution of these sub-types in the different quarters of individual cows, among cows of a given herd and among herds in general, and to discuss whether there is any difference in the extent of infection or disease in accordance with the type present. Data have been obtained for 52 herds containing, at the time of examination, 2293 cows of which 823 (35.9 per cent) were infected with *Str agalactiæ* in one or more quarters, an average of a little over two quarters each. The herds were unselected except that the owners had, in most cases, experienced some degree of trouble from mastitis, while in a few herds there had been actual outbreaks. The majority were self-contained or only restricted purchases had been made.

## Methods

The methods used were direct slide agglutination, agglutinin absorption and precipitation using acid extracts. Details have been given previously (Stableforth, 1932, 1937). Slide agglutination tests with sera for the 5 sub-types were made with one or more strains from each quarter and a clear indication was generally obtained that the strain belonged to one or other sub-type. In the earlier stages of the investigation, all strains were also examined by agglutinin absorption and precipitation tests. The absorption tests showed in nearly all cases that the indication of the slide test had been correct. The precipitation tests usually gave parallel results.

but did not as a rule distinguish between sub-types of the same main type. As work proceeded, it became clear that if a cow was infected in more than one quarter the type was in nearly all cases the same. In many herds, therefore, if the strains from different quarters of the same cow gave similar results in the slide test, absorption and precipitation tests were made with only one strain from each cow. At a later stage it became evident that the strains from a given herd were commonly alike. In certain of the later herds, therefore, when all or a large proportion of the strains from a given herd gave similar direct agglutination results, only representative strains were selected for absorption or precipitation tests. A large proportion of strains reacted in the slide test with the serum of only one sub-type. Strains which did not give clear-cut results with this test were always examined by the agglutinin absorption method. In a few herds no confirmatory absorptions were made and the strain was recorded as of a certain main type but unknown sub-type, these, however, were usually herds in which the number of infected cows was so small as to make them of little or no value for assessing the purity of herd infections. They are amongst those excluded from consideration in the section dealing with the similarity of strains obtained from the same herd and in the subsequent discussion. In most cases the small number of strains tested gave similar slide results. Altogether agglutinin absorption tests were made with strains from 867 quarters of 548 cows and precipitation tests with strains from 363 quarters of 204 cows.

As a rule one typical colony from each quarter was examined since it was already known (Stableforth, 1932) that, where a large number of colonies of apparently similar nature were present on blood agar plates sown from udder secretion, one colony could be taken as representative. With 234 quarters, however, from 2 to 6 colonies were examined, in only three cases were streptococci of two types obtained.

Strains were usually examined at once, colonies being picked direct into a sufficient quantity of medium. If not they were tested at the first sub-culture. Only one examination of most animals was made but it will be shown that change of type in a given quarter or animal is rare. 973 strains were examined by the recognised biochemical and other tests and all but a very small proportion were typical of *Str. agalactiae* in every respect. A group precipitation test was also carried out in many cases and the result clearly showed that the strains belonged to streptococcus group B (Lancefield, 1933).

#### *Stability of types and their persistence in the udder*

125 quarters were examined 2-4 times at intervals of 2-13 months. Of these, 46 were infected with sub-type 1a, 6 with 1b, 39 with 2a, 31 with 3a and 3 with 3b. In only 4 cases was a change of type found. In one of these a different type first appeared in a previously clean quarter of the same cow and, 6 months later, had apparently replaced the original type in the quarter first infected. In the other three cases a type different from the original was found after an interval of 3-6 months but there was no evidence to suggest whether there had been an actual reinfection or a change of type.

Repeated examinations have also been made in 24 quarters of cows and in halves of the udder of goats which had been artificially infected with one or other type of *Str. agalactiae*. Strains from

these animals were examined, at intervals of from less than a week to several months, for periods up to more than a year, involving, in one or two cases, a dry period and subsequent parturition. In no case was any change of type observed. In a few instances the results given by individual strains were not clear-cut but in each case the characters of the strains obtained at the next examination were identical with those of the original infecting strain.

*The distribution of the several types and sub types in cows,  
in herds and among herds*

The total number of infected cows in the 52 herds was 823 and these were infected in 1752 quarters, but for various reasons strains could be obtained from only 1546 quarters of 782 cows. It is not possible to reproduce these results in full but the complete data have been lodged in the general library of the British Museum (Natural History Section). They are briefly summarised in the sections which follow.

*The similarity of strains obtained from different quarters of the same cow.* In most cows only one sub-type was obtained. Thus of 782 cows,

641 (82.0 per cent)	)	carried one sub-type only,
57 (7.3	)	one main type, but the sub-type was not determined,
18 (2.3	)	different sub-types of the same main type,
28 (3.6	)	two or more main types,
10 (1.2	)	a main type and an unknown type,
28 (3.6	)	only strains of unknown types

*The distribution of sub-types among cows with pure infections.* Of the 641 cows with only one sub-type,

279 (43.5 per cent)	)	carried sub-type 1a,
21 (3.3	)	1b,
61 (9.5	)	2a,
224 (35.0	)	3a,
56 (8.7	)	3b

*The similarity of the strains obtained from the same herd.* In table I the data for 45 of the 52 herds are arranged according to the percentage of total infected cows in the herd, and the percentage of cows infected with one sub-type or one main type where this was clear, but no attempt was made to determine the sub-type. The remaining 7 herds were excluded because less than 4 cows in each were examined.

It is seen that in 38 of these 45 herds, at least 70 per cent of the individual infections within the herd were alike and that in 15

TABLE I  
*Incidence and purity of infection in 45 herds*

Percentage of infections within the herd due to one type or sub type	Proportion of infected cows in herd			Totals
	40 per cent, or more	20-39 per cent	Less than 20 per cent	
100 (15 herds)	1a in 2 herds* 3 (? sub) in 1 herd †	1a in 6 herds 1 (? sub) in 1 herd 3b in 1 herd 3 (? sub) in 1 herd	1a in 2 herds 1b " 1 herd	1a in 10 herds 1b " 1 herd 1 (? sub) in 1 herd 3b in 1 herd 3 (? sub) in 2 herds
80-99 (8 herds)	1a in 2 herds 3a " 3 "	1a in 3 herds	None	1a in 5 herds 3a " 3 "
60-79 (6 herds)	2a in 1 herd 3 (? sub) in 1 herd	1a in 2 herds 1b " 1 herd	1a in 1 herd	1a in 3 herds 1b " 1 herd 2a " 1 " 3 (? sub) in 1 herd
50-40 (4 herds)	1a in 1 herd 2a " 2 herds 3a " 2 " 3b " 3 "	1b in 1 herd	None	1a in 1 herd 1b " 1 " 2a " 2 herds 3a " 2 " 3b " 3 "
30-20 (3 herds)	2a in 1 herd	2a in 1 herd 3a " 1 "	None	2a in 2 herds 3a " 1 herd
Less than 60 (1 herd)	None	3 herds	1 herd	

\* "1a in 2 herds" means that sub type 1a was the predominant serological type in 2 herds

† "3 (? sub) in 1 herd" means that type 3 was the predominant serological type in 1 herd but no attempt was made to determine the sub type

of the 38 all were alike, while in the remaining 7 herds no sub type was predominant. Purity or relative purity of serological type appears therefore to be usual.

*The distribution of the various types among herds.* In table II are summarised the data for the 38 herds in which 70 per cent or more of cow infections were alike. It is seen that sub-type 1a was predominant, accounting for the infection in 19 herds, the remaining sub-types being more or less evenly distributed. When related sub-types are grouped, it is found that type 1 was responsible in 23 herds, type 3 in 12 and type 2 in only 3. Reference to table I will also show that in herds with the greater purity of serological type, the various sub-types of type 1, particularly 1a, were even more predominant.

TABLE II

*Distribution of serological type among herds and relation to incidence*

Proportion of infected cows in the herd			Totals
40 per cent or more	20-30 per cent	Less than 20 per cent	
1a in 5 herds 2a " 3 " 3a " 5 " 3b " 3 " 3 (? sub) in 2 herds } 10	1a in 11 herds 1b " 2 " 1 (? sub) in 1 herd } 14 3b in 1 herd 3 (? sub) in 1 herd	1a in 3 herds } 4 1b , 1 herd }	1a in 19 herds 1b " 3 " 1 (? sub) in 1 herd } 23 2a in 3 herds 3a " 5 " 3b " 4 " 3 (? sub) in 3 herds } 12

For meaning of terms see footnote to table I

*Incidence of infection in herds and its relation to the serological type present.* Considering the percentage of infections in the herds in relation to the infecting type (table II), it is seen that type 1 (usually 1a) accounted for all four herds with less than 20 per cent incidence and for most of those with 20-39 per cent incidence. On the other hand, type 3 was responsible in over half of the herds where the incidence was above the average, the remainder being distributed between types 1 and 2. All of the type 2 herds were in this category.

Consideration of the data from the 14 herds excluded from this analysis has shown that the incidence of types 1 and 2 was somewhat greater amongst these herds, but that their inclusion would not have altered in any important manner the proportion of herds showing a predominant type, the incidence of the various types or sub-types among herds or the relative predominance of type 1 in herds with little infection and of type 3 in herds with much infection.



*The hæmolytic activity of the strains.*

In the summary given no distinction is made between cows which carried  $\beta$  hæmolytic streptococci and those which carried non-hæmolytic streptococci because it has already been shown (Stableforth, 1932) that streptococci of both varieties may exist in the same serological type. The division of the strains in the table would moreover have confused the issue. The distribution of hæmolytic properties was as follows.

(a) Of the total cows examined, 74 per cent carried pure  $\beta$  hæmolytic infections and 23 per cent pure non-hæmolytic infections, while 3 per cent had mixed infections.

(b) Nearly all quarters carried either pure  $\beta$  hæmolytic infections or pure non-hæmolytic infections but in a few  $\beta$  and non-hæmolytic colonies of the same serological type were found.

(c) Nearly three-fifths of the herds had relatively pure infections with  $\beta$  hæmolytic strains (20 herds) or non-hæmolytic strains (9 herds), while the remainder had mixed infections.

It has already been shown that in 38 herds one sub-type only was present or one type was predominant. In nearly three-fourths of these 38 herds all or nearly all strains of the predominant sub-type showed similar characters in respect of hæmolysis, while in the remaining fourth the predominant sub-type contained both  $\beta$  hæmolytic and non-hæmolytic strains. In herds with more than one serological type both  $\beta$  and non-hæmolytic strains often occurred in the same type, on the other hand in a few herds all or nearly all the  $\beta$  hæmolytic infections were of one type whereas all the non-hæmolytic infections were of another.

*The use of serological methods for the routine identification of Str. agalactiæ*

The experience gained in the work here reported has shown that serological methods offer a ready and reliable means of recognising *Str. agalactiæ* and these have, during the past three years, gradually replaced biochemical methods in the control of *Str. agalactiæ* infections. Suspected colonies are picked into 30 c.c. of serum broth and each streptococcal growth, after removal of 1 c.c. to another tube for further use if necessary, is centrifuged and the deposit taken up in 0.5-1.0 c.c. carbol-saline. A slide agglutination test, occupying one minute, is then made with the five sera representative of most British strains. A definite reaction with any of these is indicative of *Str. agalactiæ* and also usually indicates the particular type. If the result is negative, cultures are prepared in 100 c.c. of glucose broth, extracted and tested by the contact method with a group serum known to be specific for *Str. agalactiæ* or *Streptococcus* group B. In this way most strains can be recog-

nised within 24 or at most 48 hours after the plates are examined Streptococci belonging to other groups from animals and man and those belonging to no known group have given consistently negative results

### Discussion

The various data reported in this paper further strengthen the view that mastitis caused by *Str. agalactiæ* is a purely contagious disease. It has been shown that the infection of a given quarter with *Str. agalactiæ* is, with rare exceptions, pure in a serological sense and that it remains pure over a period of at least 12 months, the last fact also connoting stability of serological type. The few occasions where a type other than the original was isolated all occurred in a heavily infected environment where reinfection or superinfection might well have taken place, and never in those animals which were isolated for experiment. It has also been shown that where a cow is infected in more than one quarter the infection is nearly always pure. Further, in most self-contained herds the infecting sub-type is the same in all or most of the cows. A specially clear case was seen on a farm where only newly calved heifers were present. In an outbreak of mastitis which occurred among these heifers, lasting about two months, the same infecting sub-type was present throughout, a significant fact since it can be assumed that these animals were, in nearly all cases, previously clean. On another farm, where regular examinations were being made, a series of new infections occurred at about the same time, all with a sub-type not previously found in the herd. That many herds, especially the larger ones, though self-contained or nearly self-contained, should show a greater diversity of type is to be expected. Nevertheless, even in these the diversity is much below what would be expected if the exposure of the cows to the several sub-types is considered to be proportional to their relative incidence in cows or herds in general.

In considering the data relative to the incidence of the various types among herds as a whole, certain points have especially to be remembered. The herds were not a random sample because in most cases the owners had complained of mastitis. Actual outbreaks occurred, however, in only a few and, in most, the amount of obvious disease was not above the average. Moreover when a clinical outbreak was observed it was generally unknown whether there had been a recent spread of infection or whether a number of previously infected cows had simultaneously shown clinical manifestations through environmental causes. Nevertheless if there is any difference in the clinical effects due to the various types—and we have some evidence suggesting that type Ia is more highly pathogenic than the others—then it is clear that some types

may be represented more often in this series than they would be in a purely random sample. It is also possible that certain types may cause a heavier bacterial infection than others or that one type may show a tendency to supplant another, both of these possibilities would increase the likelihood of a given type being isolated. Again, a type such as 3a, which usually produces  $\beta$  hæmolytic colonies, would be less likely to be missed if present in small numbers than one which is often non-hæmolytic, such as type 1. There is no reason for thinking, however, that any of the possibilities mentioned in the last two sentences have seriously biased the results. Finally, since herd infections are usually more or less pure and since the number of cows infected with any given type is therefore influenced by the relative sizes of the herds, the relative incidence of the several types must be considered in terms of herds and not of cows, and this of course considerably restricts the data available for analysis.

When these considerations are borne in mind, there is still no reason to doubt that, in British herds of the kind dealt with, type 2 is relatively uncommon, and it is also probably correct to say that type 1 (usually 1a) is responsible for the infections in nearly two-thirds of the herds and one or other of the sub-types of type 3 in the remaining third. Consideration of the data in various ways substantiates these conclusions. There is also a clear suggestion that type 3 is capable of spreading more quickly or extensively in herds than type 1, because in herds in which type 3 was predominant the incidence of infection was usually above the average, whereas in herds in which type 1 was predominant or the only infecting type, the incidence of infection was more often average or less than average.

The discussion concerning incidence among herds has been confined to the main types with little reference to sub-types because such division would have unduly reduced the size of the groups. It may be remarked, however, that sub-type 1a was wholly or mainly responsible in half of the herds.

Another point worthy of mention because it emphasises the predominance of a single type in cows and in herds is the fact that, where the herd type or sub-type showed, as was often the case, non-specific cross reactions these were remarkably similar in nature and degree.

The work here reported has emphasised the value of the slide agglutination method for recognising strains of *Str. agalactiae*. Once sera are available, results can be quickly obtained and, though a group precipitation test is also necessary if the slide test is negative, the serological method saves much time and material as compared with the usual biochemical or other tests.

### Summary

Observations have been made on the serological types of *Str. agalactiæ* isolated from 1546 infected quarters of 782 cows in 52 herds

In nearly all quarters and in most cows only one sub-type was found

Of the pure infections in cows, about 10 per cent were due to type 2, the remainder being almost equally distributed between type 1 (usually 1a) and type 3 (usually 3a). Four per cent of the cows carried unknown types

In 38 out of 45 herds 70 per cent or more of the individual infections were alike and in 15 all were alike. Of these 38 herds 23 were infected with type 1, 3 with type 2 and 12 with type 3

In herds with a low or average incidence the infecting type was nearly always type 1 (usually 1a). In over half of the remainder it was type 3

Details are given of the hæmolytic activity of the strains, and of the relation of hæmolysis to serological type

It is shown that the use of serological methods has greatly simplified the routine diagnosis of *Str. agalactiæ*

The significance of the facts reported is discussed in relation to the contagiousness of infection with *Str. agalactiæ*

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# THE CLASSIFICATION OF STAPHYLOCOCCI BY PRECIPITATION AND BIOLOGICAL REACTIONS

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WHILE Dudgeon and Simpson (1927-28) were unable to correlate the results of precipitation reactions with cultural characteristics, Julianelle and Wiegand (1935), using crude bacterial extracts or purified carbohydrates as antigens in precipitation systems, divided 16 strains of staphylococci into two groups, the pathogenic and the saprophytic. An attempt has been made to confirm the work of Julianelle and Wiegand on a larger number of strains and to correlate the findings with a classification based on metabolic activities, such as that applied to the different groups of streptococci.

## MATERIALS AND METHODS

**Source of strains** (a) **Human strains** The majority of strains were isolated from material sent for bacteriological examination. Dr Bertha Lewis of University College Hospital provided strains from cases of impetigo and Dr Julianello kindly sent two strains of each of his types.

(b) **Animal strains** Most of these were supplied by Dr R Lovell, while strains from hens were isolated from faeces obtained through Mr C A McGaughey. The mouse strains were isolated from superficial lesions.

(c) **Miscellaneous strains** Strains isolated from food and thought to be implicated in outbreaks of food poisoning were given to me by Dr W V Scott of the Ministry of Health, and Dr H J Shaughnessy of Springfield, Illinois, kindly sent me a food poisoning strain isolated from milk. A strain isolated from water is included in this group.

**Pigment production** Pure cultures were grown on agar slopes at 37° C for 24 hours and left at room temperature for a week. Pigment of cream colour has been included with golden yellow.

**Mannitol fermentation** Strains were grown in peptone water containing 0.5 per cent mannitol and Andrade's indicator. The majority of strains fermenting this sugar produced acid within a few days but occasional strains have required incubation up to 16 days.

**Gelatin liquefaction** Stab cultures were kept at room temperature (15-23° C) for a month or more and liquefaction noted.

**Loges-Proskauer reaction** Seven day cultures in dextrose phosphate medium were examined by two methods, (a) the addition of 40 per cent KOH, and (b) the α-naphthol modification described by Barritt (1936). Experiment showed that only a few strains negative at 7 days became positive after longer incubation.

*Hæmolysin production* Strains were grown on 0.3 per cent nutrient agar for 40-48 hours in 25-30 per cent  $\text{CO}_2$ . After centrifugation to remove agar and the majority of the organisms, dilutions were prepared in saline and duplicate tests made by adding 0.5 c.c. of these to 1.0 c.c. of 2 per cent suspensions of rabbit and sheep cells. Readings were taken after 1 hour at  $37^\circ\text{C}$  and 2 hours at room temperature. For the sake of brevity strains producing hæmolysis of rabbit cells have been called  $\alpha$  hæmolysin producers, although in only a few instances have neutralisation tests been made with  $\alpha$  antitoxin. Strains showing the hot-cold lysis of sheep cells (Bigger, Boland and O'Meara, 1927) have been called  $\beta$  hæmolysin producers, lysis of sheep cells in low dilution but not showing the hot-cold phenomenon has been considered to be due to the  $\alpha$  hæmolysin. Strains producing hot-cold lysis of sheep cells and lysis of rabbit cells have been labelled  $\alpha\beta$  hæmolysin producers since Bryce and Rountree (1936) have shown that  $\beta$  hæmolysin has little effect on rabbit cells.

*Coagulase* All tests were made using one of two samples of citrated human plasma, the method was either to mix equal volumes of 24-hour broth culture and plasma, or to add a loopful of agar-grown culture to a 1:2 dilution of plasma. The mixtures were placed at  $37^\circ\text{C}$  and the results read after 4 hours.

## RESULTS.

The association of the biological characters of human and animal strains is shown in tables I and II respectively, while table III shows the combined results of the human, animal and miscellaneous strains of staphylococcus.

*Mannitol fermentation* With one exception all human golden yellow strains fermented mannitol. The association of hæmolysin production and mannitol fermentation was greater with human (94 per cent) than with animal (68 per cent) strains. No example of a strain producing a reversion to alkalinity (Julianelle, 1937) was found.

*Gelatin liquefaction* Only 15 of 146 strains failed to liquefy gelatin and 13 of these were white.

*Voges-Proskauer reaction* The different results obtained by the use of the two methods appear to be due to the greater sensitivity of the  $\alpha$ -naphthol modification. No strains were found positive by the older method and negative by the modification. Human strains were mainly V.P.-positive (94.7 and 80.7 per cent by the  $\alpha$  naphthol and older method respectively), there were fewer V.P.-positives among the animal strains (45 and 40 per cent) and further analysis showed that all the strains from mice (3 strains), hen faeces (3), dogs (4) and guinea-pig (1) were V.P.-negative, while strains from bovines (4), horses (3), sheep (1) and rabbit (1) were V.P.-positive. The number of strains from each species is small but the results suggest that the V.P. reaction might be helpful in the subdivision of animal strains.

*Hæmolysin production* All human golden yellow strains produced hæmolysin but 6 non-human strains—3 of animal origin—





TABLE II  
*Biological reactions of animal strains of staphylococci.*

[illegible]



failed to do so: two lemon-yellow strains of human origin also produced hæmolysin. Of the white strains rather more than two-thirds failed to produce hæmolysin.

Of the human strains only 3 produced  $\beta$  hæmolysin and since they produced  $\alpha$  hæmolysin in addition they were placed in the  $\alpha\beta$  group. Among the animal strains only 2 produced  $\alpha$  hæmolysin alone, while of the 11 producing  $\beta$  lysin, only 4 failed to produce  $\alpha$  lysin, these 4 strains were from dogs.

**Coagulase** All strains producing either  $\alpha$  or  $\beta$  hæmolysin or both were coagulase-positive and all strains failing to produce hæmolysin were coagulase-negative. There was thus an absolute correlation between hæmolysin ( $\alpha$  and  $\beta$ ) and coagulase production.

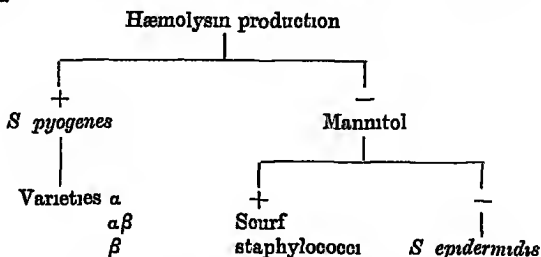
### *Classification of strains on metabolic activities*

**Human strains** Using eight differential tests Andrewes and Gordon (1907) divided staphylococci into four species. Certain of their tests could be eliminated and the species defined by the consideration of pigment production, mannitol fermentation and gelatin liquefaction. Using these criteria all the human strains of this series with one exception (a golden yellow non-mannitol-fermenting strain) could be classified, but three non-hæmolysin-producing strains would be included in the *pyogenes* group. In the light of present knowledge hæmolysin production is considered to be of major importance in the classification of staphylococci and for the purpose of this paper the *pyogenes* species of Andrewes and Gordon has been modified to include only those strains which produce hæmolysin. The golden yellow non-mannitol-fermenting strain mentioned above differed from the *pyogenes* species only in its inability to ferment mannitol, and as it produced  $\alpha$  hæmolysin it has been included in this species. It is proposed to subdivide the species *Staphylococcus pyogenes* into three varieties according to the type of hæmolysin produced; these varieties will be indicated by the suffixes  $\alpha$ ,  $\alpha\beta$  and  $\beta$ .

The three strains which fell into Andrewes and Gordon's *pyogenes* group, but are excluded from the re-defined species on account of their failure to produce hæmolysin, differ from the scurf staphylococci only in their ability to liquefy gelatin. It is proposed that these mannitol-fermenting, non-hæmolysin-producing strains should be placed provisionally with the scurf staphylococci, as gelatin liquefaction appears to have little taxonomic value. For the same reason the gelatin-liquefying and non-liquefying strains which fail to ferment mannitol (*epidermidis* and *salivarius* species of Andrewes and Gordon) have been combined and the specific name *epidermidis* is used to indicate this group.

This method of classification of staphylococci which we suggest

and which will be used in this paper is indicated in the accompanying schema



**Animal and miscellaneous strains** The strains of animal and miscellaneous origin can be divided among the species defined for human strains. Tables II and III show that certain pigment-producing strains of non-human origin do not produce haemolysin but, as pigment production has not been taken into account in the classification, these strains have been allocated to species from which they would have been excluded by adherence to Andrewes and Gordon's criteria.

Table IV shows the allocation of the strains among the re-defined species and also the association of the characters not considered in the division into species. It is clear that a classification based on pigment production would have given a very different result: there would have been 93 *aureus* strains and of these 6 would be non-haemolysin producers, while of 48 *albus* strains 14 would have produced haemolysin. It is interesting that of 12 scurf strains only one was V P -positive when tested by the usual method.

### Serological investigations

**Immunisation of rabbits** Heat killed saline suspensions prepared from broth cultures were inoculated intravenously on three successive days each week, every fourth week rabbits were rested and bled for trial purposes. Immunisation for 3-6 months was usually required to produce sera suitable for precipitation reactions. The response to one strain varied in individual animals but, contrary to Julianelle and Wieghard's experience, this type of variation was less common than that of the response to different strains of staphylococcus. In agreement with Dudgeon and Simpson a feeble response was obtained with saprophytes and some pathogenic strains appeared to be weakly antigenic. Sera were stored over chloroform in the refrigerator and usually kept well for over 12 months.

At the beginning of the investigation some rabbits were immunised with a strain of *Staph. pyogenes* α (S 33) isolated from a pleural effusion and others with a strain of *Staph. epidermidis* (S 34) isolated from healthy skin. These were called "A" and "B" strains respectively in accordance with the terminology of Julianelle and Wieghard, their serological relationship to the strains of these workers was subsequently confirmed. For



reasons to be discussed later the word "group" is used instead of "type" in describing strains showing serological relationship with one another

Since the antigens prepared from certain strains of *Staph. pyogenes* did not react with the group A sera available, additional rabbits were immunised with another member of group A and with three of the strains which failed to react with group A sera. Rabbits were also immunised with a scurf staphylococcus (S 41) which appeared to be serologically distinct from group A or B strains. This strain—S 41—was provisionally labelled group D.

**Preparation of antigen** In the earlier experiments extracts were prepared by heating suspensions of staphylococci in acid and neutralising with alkali, the method used by Lancefield for streptococci and by Julianelle and Wiegand for staphylococci. Experiments were made to find a method which would extract the antigens in a more natural state. Suspensions of staphylococci in distilled water were kept in the refrigerator and at 37° C for prolonged periods and emular suspensions were heated at various temperatures for shorter periods. Prolonged extraction at 4° or 37° C yielded extracts giving considerable cross reactions, while boiling for 12 hours or autoclaving at 120° C for 15 minutes gave extracts which, on the whole, were much more specific. In the later part of the work boiling or autoclaving aqueous suspensions of agar grown organisms has been employed. In all cases the antigen solutions have been clarified by centrifugation.

**Concentration of antigen** Considerable variation has been found in the amount of soluble antigen extractable at different times from any one strain (see table VI) and in many cases it has been necessary to concentrate the antigen in order to determine its serological reactivity. This was done by precipitating with 5 or 6 volumes of alcohol and taking up the precipitate in a small volume of saline. Partial purification has sometimes been made by precipitation with acid and reprecipitations with alcohol. In the whole series it was necessary to prepare concentrated antigens of 48 strains (including 21 unclassified strains) before the serological reactions could be studied satisfactorily.

**Precipitation reactions** Sera were diluted in saline (usually 1:10) and 0.25 c.c. placed in a series of small tubes. Antigen dilutions (threefold in a preliminary orientation) were made in saline and were added in equal volume. The tubes were shaken and placed in a water bath at 56° C. Readings were made after four hours and after standing over night at room temperature. Ring tests were made occasionally in narrow tubes, particularly when it was desirable to economise absorbed sera.

### *Precipitation reactions of 157 strains of staphylococcus*

Antigens were prepared from the 146 strains described in the previous section and from 11 additional strains of *Staph. pyogenes* whose biochemical reactions had not been completely studied. The method adopted was to put up each antigen against group A and group B sera and if no reaction was obtained to test it with sera prepared against other strains. Strains which could not be classified by this procedure were reinvestigated by means of a fresh preparation of antigen, concentrated if necessary. A number of strains could be classified by the use of two sera, a smaller number had to be put up against additional sera, while absorption had to be resorted to when antigens reacted with sera of more than one

group The main difficulty experienced was with certain group B sera which reacted with antigens of group A strains it was possible however to absorb out the cross-reacting antibody with a group A antigen, leaving precipitins for group B strains almost intact

In table V are summarised the results of an experiment in which extracts of 7 strains were put up against 4 different antisera, the maximum precipitation observed is shown, irrespective of the antigen dilution The group C serum was prepared by immunising a rabbit against a strain of *Staph pyogenes* a (S 8) which reacted feebly or not at all with group A sera This serum was unsatisfactory in that it reacted only feebly with extracts of the homologous strain, and absorption with an extract of S 11 (group A) removed precipitins for the homologous strain S 8 as well as for group A strains Since the strain S 8 appeared to be a

TABLE V

*Maximum precipitation between various antigens and four antisera*

Strain used as antigen	Serological group	Serum			
		Group A (S 33)	Group B (S 34)	Group C (S 8)	Group D (S 41)
S 11	A	++++	++++	++++	—
S 33	A	++++	—	+	—
S 34	B	—	++++	—	—
S 211	B	—	++++	—	—
S 8	C	+	+	++	—
S 80	C	+	+	++	—
S 41	D	—	—	—	++++

+ to + + + + = increasing degrees of precipitation

— = no reaction

poor antigen, rabbits were immunised with a strain S 80 which was thought to be antigenically similar A trial bleeding of one of the rabbits yielded a serum which reacted strongly with the homologous antigen The reactions obtained with this serum and two group A sera with extracts of S 11, S 33 and S 80 are recorded in table VI

With serum 36 (group A) the extract of S 80 gave a small compact granular precipitate which was quite different from the usual bulky flocculation seen in these reactions Julienne and Wiegand noticed the formation of granular precipitates with heterologous antisera and it is suggested tentatively that such reactions may be due to some species antigen and antibody Table VI also shows the different reactions obtained with two extracts of S 11 and differences between S 11 and S 33. whether the latter are greater than those observed with different preparations of the same strain is uncertain The reactions as a whole can

be explained by postulating either that all strains have a species antigen in common and that S 11 and S 80 have different group antigens, or that S 11, S 33 and S 80 have each at least two antigens in common but in varying proportions. If the antigens were named  $x$  and  $y$  then S 11 could be represented as having  $x$  and  $y$  in equal proportions, S 33 having  $x$  as the major antigen and only a small amount of  $y$ , while S 80 would have mostly  $y$  and a smaller amount of  $x$ .

TABLE VI

*Precipitation reactions between extracts of three strains of Staph. pyogenes  $\alpha$  and antisera prepared against them*

Extract of strain	Serum (strain used for immunisation indicated in brackets)		
	66 (S 33)	B 143 (S 11)	B 184 (S 80)
S 11a	+++ +	++++	++++
S 11b	++	+++	+
S 33	++++	+++	—
S 80	++ G	++ +	++++

a and b = different extracts of S 11

G = compact, granular precipitation

It is not proposed to discuss the antigenic structure further, as the work at this stage does not justify any definite conclusions being made. The relationship and differences between S 11, S 33 and S 80 have been introduced to justify the tentative assignment of strains resembling S 80 to a group distinct from A. The slide agglutination technique enables clear differentiation to be made between these strains, and sub-groups or "types" can also be determined by this method. Further consideration of this method will be deferred until a larger number of strains has been examined.

Antigens of certain other strains of *S. pyogenes*  $\alpha$  were not precipitated by sera of groups A, B, C or D, or reacted only feebly with group A or C. These strains have been placed provisionally with the group C strains, a procedure which seems justified until the subdivision of these strains and their relationship to group A strains is clarified by further work.

Antigens of strains of species other than *S. pyogenes*  $\alpha$  and *S. pyogenes*  $\alpha\beta$  which have not been classified by precipitin reactions have been collected into a heterogeneous group X.

#### *Source of strains and serological grouping*

In table VII the source of the 157 strains is correlated with the serological grouping obtained by means of the precipitation reaction. Under the heading of "urine, infected" are classed those strains which were present in sufficient numbers to be seen on direct examination of smears of catheter specimens, generally



in more than one specimen from the same patient Those included under "urine, contaminated" could not be found in the urine in this way

TABLE VII

*Serological grouping of 157 strains of staphylococci from various sources.*

Source	Serological group					Remarks
	A	B	C	D	X	
<b>Human</b>						
Skin, healthy	0	1	0	0	2	Includes boils and carbuncles
„ infected	22	3	6	1	1	
Conjunctiva, normal	0	1	0	0	1	*Gonococcal conjunctivitis
„ infected	5	1*	1	0	1	
Vagina and cervix	3	2	0	1	1	
Nose and throat	16	1	0	0	0	
Pus	29	3	8	0	1	
Urine, contaminated	0	7	0	0	2	
„ infected	3	1	0	1	0	
<b>Total human strains</b>	<b>78</b>	<b>20</b>	<b>15</b>	<b>3</b>	<b>9</b>	
<b>Other sources</b>						
Dog	0	3	0	0	1	
Horse	3	0	0	0	0	
Cow (including milk)	6	0	0	0	0	
Sheep (1), rabbit (1)	2	0	0	0	0	
Guinea-pig (1), mice (3), hen (3)	0	0	0	0	7	
Foodstuffs etc	6	0	0	0	4	
<b>Total animal and miscellaneous</b>	<b>17</b>	<b>3</b>	<b>0</b>	<b>0</b>	<b>12</b>	
<b>Grand total</b>	<b>95</b>	<b>23</b>	<b>15</b>	<b>3</b>	<b>21</b>	

Strains of animal origin fell mainly into group A but 3 of the 4 dog strains were group B and one strain was unclassified Gibbs (1936) was able to differentiate avian from human strains by means of precipitin tests and 3 avian strains in this series did not react with sera prepared against human strains

Of the 95 strains in which A was the predominant antigen 77 were classified by testing crude extract against two sera only With the other strains either concentration of antigen or absorption of sera or both was necessary

*Correlation of the biological and serological classifications of staphylococci*

The relationship of the serological classification with that described in the first section of this paper is shown in table VIII

Strains falling into group A and the provisional group C were all  $\alpha$  haemolysin producers while 12 produced  $\beta$  haemolysin in addition. It should be pointed out that the reaction in these groups was not between toxin and antitoxin since the majority of the antibacterial sera did not contain  $\alpha$  antitoxin above the

TABLE VIII

Correlation of the biological and serological classifications of staphylococci

	Serological group				
	A	B	C	D	$\gamma$
<i>S. pyogenes</i> $\alpha$	84	0	14	0	0
<i>S. pyogenes</i> $\alpha\beta$	11	0	1	0	0
<i>S. pyogenes</i> $\beta$	0	3	0	0	1
Scurf staphylococci	0	3	0	2	7
<i>S. epidermidis</i>	0	17	0	1	13

normal level (Downie, 1937). Group B comprised 3 canine strains of *S. pyogenes*  $\beta$  and 20 saprophytic strains of human origin. Only 3 strains fell into group D, one each from infected urine, vulvitis and the cervix. The group of unclassified strains contained apparently saprophytic strains of human origin and a number of animal strains apart from the canine strain of *S. pyogenes*  $\beta$ . It is uncertain what part these unclassified strains of animal origin played in the morbid processes from which they were isolated. The avian strains were from the faeces of apparently normal fowls.

#### DISCUSSION AND SUMMARY

A classification based on that of Andrewes and Gordon but modified chiefly by the inclusion of haemolysin production has been used in a subdivision of staphylococci. Since it is doubtful if much is to be gained by a subdivision of the non-haemolysin-producing strains into different species, the *epidermidis* and *salvarius* species of Andrewes and Gordon have been combined under the specific name *epidermidis*, while all non-haemolytic strains which fermented mannitol have been included with the scurf staphylococci irrespective of the liquefaction of gelatin. The slight taxonomic value of gelatin liquefaction in this series may possibly be explained by the fact that only a qualitative difference was recorded, no account being taken of the rate of liquefaction.

The haemolysin for rabbit cells probably corresponds to  $\alpha$  toxin, although in certain strains it may be the  $\gamma$  haemolysin described by Smith in a communication to the Pathological Society of Great Britain and Ireland in January 1937. Morgan and Graydon (1936) have separated  $\alpha$  toxin into two components, both of which

hæmolyse rabbit cells : of the toxigenic strains examined by them all produced  $\alpha_1$  toxin (the toxin neutralised by standard antitoxin) while about two-thirds produced  $\alpha_2$  toxin. The hot-cold lysis of sheep cells appears to be a property of  $\beta$  toxin (Glenny and Stevens, 1935, Bryce and Rountree, 1936) of the 16 strains showing this phenomenon only 3 were of human origin and all three produced rabbit cell lysin in addition.

Examination of the metabolic activities has shown an absolute correlation between hæmolysin ( $\alpha$  and  $\beta$ ) and coagulase production as suggested by Cruickshank (1937) the latter appears to offer the simplest criterion for pathogenicity as understood at the present time.

The precipitation reactions showed that the majority of strains of *S. pyogenes*  $\alpha$  and *S. pyogenes*  $\alpha\beta$  fell into one serological group. There were, however, 15 strains which could not be classified satisfactorily with the sera available. For the moment these have been grouped together, although there is a possibility that this group will prove heterogeneous on further study. Non-hæmolysin-producing staphylococci appear to be more heterogeneous serologically, while 20 of these strains could be placed in one group and 3 into another, the remaining 20 strains were not classified.

The assertion of Julianelle and Wieghard that staphylococci can be divided into two groups by precipitation reactions requires modification. This has already been suggested by the work of Thompson and Khorazo (1937), who found evidence for at least one other group among the non-pathogenic staphylococci, and the work reported here indicates that further subdivision of the pathogenic strains may be possible.

Numerous workers including Hine (1922) and Julianelle (1922) have shown that staphylococci can be divided into a number of serological types by means of agglutinin absorption. It has been found by slide agglutination that *S. pyogenes* can be divided into at least three distinct types and possibly several sub-types showing only minor differences in antigenic structure. Saprophytic strains are much more heterogeneous when examined by this technique. In conformity with the terminology used in connection with the streptococci, it is suggested that the division made by the precipitin reaction should be defined as a series of "groups" leaving the way open for further subdivision into "types". Thus the type A of Julianelle and Wieghard would become group A. Of the strains received from Julianelle, both his A strains fell into group A of this investigation but antigens of only one of his B strains were precipitated by the group B sera; extracts of the other B strain received were not precipitated by any of the sera so far prepared.

Confusion of results due to agar precipitins (Morgan, 1936) has been avoided by using only suspensions of broth-grown organisms.

for the immunisation of rabbits Multiplicity of antigens in staphylococci and phage-resistant variants has been shown by Burnet and McKie (1929) and in the experiments recorded here the cross reactions shown by certain sera suggest either that these sera possess species in addition to group antibody or possibly that there is among staphylococci a sharing of antigenic constituents in varying proportions similar to that postulated for organisms of the Brucella group by Wilson and Miles (1932)

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# EXPERIMENTAL SYMMETRICAL CORTICAL NECROSIS OF THE KIDNEYS PRODUCED BY STAPHYLOCOCCUS TOXIN A STUDY OF THE MORBID ANATOMY AND ASSOCIATED CIRCULATORY AND BIOCHEMICAL CHANGES

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(PLATES II-IV)

RENAL changes in rabbits following the injection of filtrates from cultures of staphylococci have been described on several occasions, but the anatomical similarity of the lesions to those of symmetrical cortical necrosis of the kidneys in human beings appears to have been overlooked. Neisser and Levaditi (1901) produced an ischaemic necrosis of the outer renal cortex in rabbits by intravenous injection of 1-5 c.c. of a filtrate from a culture of staphylococcus which was rich in haemolysin and leucocidin. They considered the lesion to be specific to staphylococcus toxin. Forssman (1932) described total necrosis of the renal cortex in rabbits following the injection of similar filtrates, while more recently VonGlahn and Weld (1935) produced similar lesions in rabbits and cats, which they ascribed to injury of the renal arterioles by the toxin of *Staphylococcus aureus*.

In a previous communication (De Navasquez, 1935) the changes present in twelve cases of symmetrical cortical necrosis of pregnancy were fully described. As a result of the histological investigations made, it was concluded that the pathogenesis of the lesions consisted of a primary toxic necrosis of the intralobular arteries and their afferent arterioles which resulted in a secondary ischaemic necrosis of the cortex supplied by those vessels. Since the renal lesions in staphylococcal toxæmia in rabbits strongly resemble those seen in human cases of cortical necrosis of pregnancy, it was hoped that the experimental study in rabbits would test these conclusions as to the priority of the vascular lesions and elucidate further the origin of the arterial necrosis.

This experimental study fell into two parts. The first, primarily morphological, was concerned with the anatomical and histological appearances in the kidneys of rabbits with staphylococcus toxæmia and the relation of these changes to those found in the kidneys of

human beings dying with symmetrical cortical necrosis of pregnancy. The second was concerned with the functional changes in urinary secretion occurring both early and late in staphylococcus toxæmia, with special reference to the bearing which these changes might have on the pathogenesis of the condition. The investigation therefore required the combination of histological studies with certain functional tests on a series of rabbits surviving for various lengths of time after the injection of staphylococcus toxin.

### *Methods*

*Animals* Young male and female rabbits of approximately 2 kg weight were kept in metabolism cages arranged for the collection of urine.

*Toxin* The toxin employed was very kindly supplied by Dr H. J. Parish of the Wellcome Physiological Research Laboratories, to whom I am indebted for the following particulars. Staphylococcus toxin B 8458, "Wood 46" strain. Contains a hæmolysin only, with an M.H.D. of 1/32 when mixed with an equal volume of a 2 per cent suspension of rabbit red cells. Combining power equivalent to 4.8 units of antitoxin per c.c. of toxin. Rabbit intravenous test: 1 c.c. kills a 2 kg rabbit in 30 minutes.

*Dosage* In order to allow the rabbits to survive sufficiently long for renal changes to develop and death to occur from uræmia, single doses of 0.2-0.4 c.c. per kg of body weight were injected intravenously. Animals either died or were killed at varying intervals after a single injection.

*Histology* The kidneys were weighed and examined histologically. Both radial and tangential serial sections were made so as to include as many vessels as possible in cross section, and also to follow any changes in the arteries throughout their course in the cortex.

*Controls* Control experiments were carried out on rabbits injected with toxin inactivated by heat, which destroyed the hæmolysin.

*Renal function* This was estimated by examination of the daily output of urine, the blood urea and the phenol red excretion test (Peters and Van Slyke, 1932). The animals were catheterised before the test and again at hourly intervals after the injection of dye.

*Blood pressure estimations* These were carried out by the method devised by Grant and Rothschild (1934), which consists of an air-tight capsule fitted over the central artery of the rabbit's ear and connected with a mercury manometer. The ear was previously denervated to ensure complete vaso-dilatation of the artery, which was then compressed by pressure from a rubber bulb connected to the capsule. This device gave a reliable measure of the systolic arterial pressure.

*Blood sugar* Blood sugar estimations were made by the Folin-Wu method.

*Estimation of iron in the kidneys* After sudden death of the rabbit, caused by an intravenous overdose of nembutal (200 mg), the abdomen was immediately opened and the renal vessels clamped. After stripping the perinephric tissues the kidneys were weighed. The left kidney was then minced and dried at 105°C and the amount of iron estimated by Fowweather's method (1926).

*Adrenalectomy* This operation was conducted under light nembutal and ether anaesthesia. The right adrenal was removed first and the left about a week later. A sufficient number of rabbits recovered from total adrenalectomy to survive for 6-10 days to allow the study of the development of staphylococcus toxin renal necrosis.

## PART I MORBID ANATOMY AND HISTOLOGY

A wide degree of variation in the susceptibility of individual animals to the same dose of toxin was encountered. Some died within a few hours of injection, presumably from the acute circulatory failure described by Kellaway, Burnet and Williams (1930), which consisted of an acute fall in blood pressure due to obstruction in the pulmonary circulation. Others died a few days later with well marked renal lesions and uræmia.

*Changes in the kidneys*

The anatomical findings in the kidneys are considered in order of time of survival.

**3½ hours** Six rabbits died and eight were killed at various intervals up to 3½ hours after injection. At autopsy all the animals had enlarged kidneys weighing up to 80 per cent more than normal. The only anatomical changes visible were in the outer layer of the cortex, which showed various degrees of patchy or diffuse congestion (fig. 2).

On histological examination the parenchyma was unaffected, but the arterial tree from the intralobular arteries of 150  $\mu$  diameter to the capillaries of the glomerular tuft was dilated and engorged (fig. 7), the internal elastic lamina appearing stretched and thinned with loss of its usual wavy outline. The intima, media and adventitia appeared normal. The lumen was densely packed with red cells, which were also very conspicuous in the glomeruli supplied by these vessels. In these glomeruli the capillaries were swollen and the finger-like projections of the normal tuft were expanded to resemble a globular mass of red cells tending to form rouleaux, but bounded by a stretched but intact endothelium. The capsular space was partially or completely obliterated, while the capsular epithelium appeared normal. These engorged glomeruli contrasted strikingly with the normal glomeruli in the unaffected cortex. Thrombosis was absent throughout the affected arterial bed, whose main feature was that of extreme dilatation. The usual separation of individual cells by plasma was absent. These changes were either confined to scattered groups of glomeruli and afferent arterioles or were present throughout the outer two-thirds of the cortex. The main branches of the renal artery appeared normal.

**3½-7 hours** Four animals died and five were killed in this period. All showed similar changes to those already described (fig. 3), while, in addition, four which were killed at 4½, 5½, 5¾ and 7 hours respectively showed histological changes in the media of the intralobular arteries and afferent arterioles (fig. 8). The media had completely lost its cellular structure and consisted of a



homogeneous eosinophilic matrix in which red cells could be clearly distinguished. This dissolution of the media was unaccompanied by any inflammatory response or necrosis of the rest of the arterial wall. The intimal endothelium appeared loose but was otherwise normal. The internal elastic lamina was stretched and minute interruptions were discernible in its continuity. The adventitia was intact. The presence of numerous red cells in the spaces between the remains of the muscle fibres of the media was the most striking feature of the arterial lesion at this stage. The vessel lumen was grossly dilated and filled with masses of red cells which also packed the capillary loops of the glomeruli. An occasional red cell could be seen in the glomerular space, though the capillary endothelium still retained its outline. Thrombosis was never observed in any vessel. The parenchyma at this stage was devoid of any marked changes. The convoluted tubules were partially dilated and filled with a clear pale eosinophilic fluid. Their epithelium appeared swollen and granular at 7 hours, but the nuclei were normal and showed no evidence of pyknosis.

**12-24 hours.** Five animals died in this period. The arterial lesions had increased in severity in that red cells could now be seen in the adventitia, which was disintegrating and causing peri-arterial hæmorrhage. The intimal endothelium had been shed and the internal elastic lamina was fragmented, though it retained its staining properties and rough outline. The red cells had begun to lose their bright red staining with eosin and had assumed a yellowish tinge. A tendency to conglutinate was noticed, especially in the glomeruli and in the centre of the lumen of the intralobular arteries and arterioles, but fibrin and platelets were absent. The epithelium of the peripheral cortex supplied by these vessels showed every stage of ischæmic necrosis, with retention of the gross tubular architecture and faint cellular outlines as in areas of early hæmorrhagic infarction. The naked-eye resemblance to red infarcts was even more marked (fig 4). The affected areas were raised, firm and red, and were confined to the outer two-thirds of the cortex. In some cases the lesion was diffuse and continuous throughout the peripheral cortex, while in others the lesions were focal and separated by areas of normal cortex. The inter-medullary cortex, boundary zone, medulla and pelvis were normal.

**24 hours-8 days.** Seven animals were killed and two died in this period. From 29 to 46 hours the necrotic areas had receded slightly and were surrounded by well defined zones of congestion (fig 5), with well marked leucocytic infiltration in the later stages. The intralobular arteries and afferent arterioles still retained their outline by reason of their elastic tissue (fig. 9), which was the most resistant element in the arterial wall and could be recognised throughout its length from the interlobular arteries of the boundary

## EXPERIMENTAL CORTICAL RENAL NECROSIS

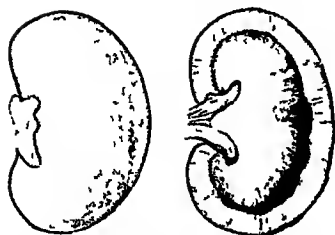


FIG. 1—A normal rabbit's kidney showing the well defined boundary zone between cortex and medulla

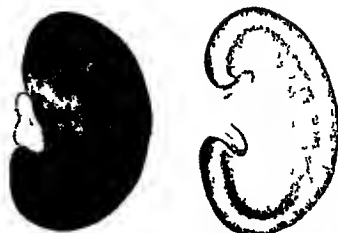


FIG. 2—Rabbit no. 7. Kidney 3 hours after the injection of staphylococcal toxin. The arteries and arterioles in the outer half of the cortex are dilated

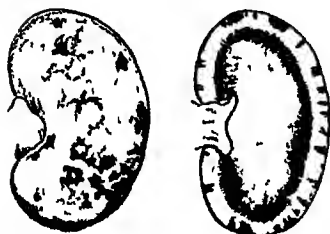


FIG. 3—Rabbit no. 10. Kidney 7 hours after the injection of toxin. The lesion in this particular animal is patchy but is still confined to the outer half of the cortex

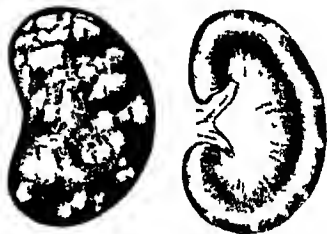


FIG. 4—Rabbit no. 1. Kidney 24 hours after toxin showing a diffuse hemorrhagic necrosis of the peripheral cortex



FIG. 5—Rabbit no. 6. Kidney 28 hours after toxin showing diffuse necrosis. This is becoming ischemic and is surrounded by a zone of congestion which is shown on the surface

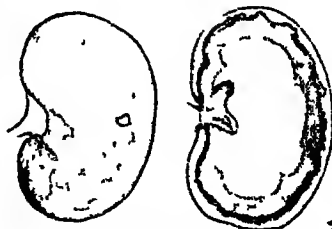


FIG. 6—Rabbit no. 9. Kidney 54 hours after toxin showing fully developed necrosis of the outer cortex which is now completely ischemic. The inner half of the cortex appears normal



## EXPERIMENTAL CORTICAL RENAL NECROSIS



FIG. 7—31 hours after injection of toxin. Kidney showing extreme dilatation and congestion of the afferent arterioles and glomeruli. H & E  $\times 60$

FIG. 8—11 hours. Renal cortex showing early changes in the media of the afferent arterioles. H & E  $\times 450$

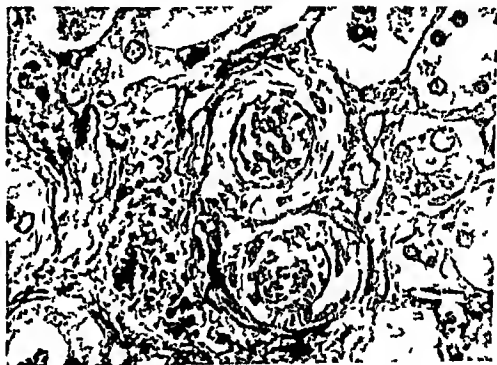


FIG. 9—24 hours. Renal cortex arteriole showing complete necrosis of the media and the presence of red cells in the medial space. H & E  $\times 600$



zone to within a few  $\mu$  of the glomerular capillaries. From the intralobular arteries of approximately 150  $\mu$  diameter to the termination of the afferent arteriole, except for a very short distance of a few  $\mu$  before the arteriole divides into the glomerular capillaries and where the elastica normally disappears, the elastic tissue could be recognised as black threads and fragments in sections stained with Weigert's elastic stain (fig 10). A few endothelial cells were still intact, though the majority had been shed. The media and adventitia were now replaced by nuclear and cytoplasmic debris in which polymorphs and macrophages were present. The lumen of the affected arterial stretch was filled in its terminal portions with conglutinated red cells (fig 10), these had fused to form solid, pale and discoloured masses in which the faint outline of red cells could still be seen at the periphery. In the intralobular arteries of increasing diameter (50-150  $\mu$ ), these conglutinated masses showed a tendency to occupy the centre of the lumen, leaving the margins free. Conglutination was also less compact in the larger arteries, where spaces between the more brightly staining red cells could be seen.

From 48 hours onwards, the complete picture of symmetrical cortical necrosis as usually seen in human beings was reproduced (fig 6). The necrotic cortex was now pale, yellow and soft and its demarcation from the normal cortex sharply defined. It was noticeable that in this group the lesion was usually of the diffuse type. The depth of the unaffected cortex from the boundary zone to the margin of the necrotic area varied in different animals, but was usually a third or less of the total cortex (fig 11). Both the vasculature and the parenchyma of this cortico-medullary zone were invariably normal. The collecting tubules in the medulla were sometimes filled with clear eosinophilic fluid, but no epithelial casts were seen at any time. All the cases in this group had a high and increasing blood urea, with oliguria, and when not killed at an earlier stage died from uræmia. One animal (no 89) lived for 8 days after a single injection of toxin and finally died with a blood urea of 670 mg per cent. This was the longest period of survival and this rabbit continued to secrete a fair quantity of urine daily. At autopsy, the kidneys, although enlarged, showed irregular depressed areas, firm and pale grey in colour, which were surrounded by zones of apparent congestion. These areas were confined to the peripheral cortex and proved on histological examination to be infarcts which were undergoing organisation. Macrophages and fibroblasts were abundant in the periphery of the necrotic areas and organisation was proceeding rapidly towards the centre. The arteries in the affected areas were undergoing a similar process of repair, their walls consisting of a thick layer of young fibroblasts concentrically arranged around a central lumen.

containing blood. It was difficult to recognise any individuality in the various layers of the arterial wall, as the encircling cells failed to show any differentiation with van Gieson's stain, although a few collagen fibrils could be seen in the position of the adventitia. Repair was evident round the entire margin of the area supplied by the affected artery. The glomeruli showed crescent formation in the capsular spaces, consisting of proliferating epithelium and fibroblasts which were invading the glomerular tuft, causing its adhesion to the capsular membrane. The histological picture bore a strong resemblance to that seen in the second stage of glomerulonephritis and denoted a similar process of repair. The intervening nephrons, which had escaped the necrosis, showed few changes from normal. The glomeruli were filled with blood and the convoluted tubules dilated. The collecting tubules contained eosinophilic granular debris only.

#### *Changes in other organs.*

The suprarenals were the only organs other than the kidneys to show lesions referable to the direct action of the toxin. An increasing proportion of animals which survived more than 12 hours showed changes in both cortex and medulla. The lesions in the cortex consisted of isolated patches of necrosis, usually confined to the periphery. These were probably ischæmic in origin, as cytoplasmic outlines were still visible and were occasionally accompanied by capillary hæmorrhage. The necrotic areas were rounded and sharply defined by a zone of leucocytes from the rest of the cortical tissue, which presented no abnormality. The extent and severity of the necrosis varied, but it seldom involved more than a small proportion of the total cortex.

The changes in the medulla were more constant and were invariably associated with the hyperglycæmia which followed the injection of toxin. By means of the staining method described by Ogata and Ogata (1917) it was possible to gauge the amount of adrenalin present in the chromaffin cells of the medulla. There was a marked reduction in the normal content of adrenalin (*cf.* figs 12 and 13). These changes could be attributed to the adrenalin release since animals which received inactivated toxin showed a normal adrenalin content. No other changes were seen in the medulla.

The liver in animals which were allowed to die sometimes showed "nutmeg" changes due to passive congestion, probably the result of a fall in arterial pressure. The bladder was usually empty, though in some animals clear urine was present in variable quantity. The other viscera, including heart, lungs, pancreas and spleen, were carefully examined for evidence of arterial disease but were always normal on histological examination. The peritoneal cavity some-

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FIG. 10—30 hours. An afferent arteriole showing the retention of the elastic tissue of the wall and conglutination of red cells in the lumen. Weigert's elastic stain.  $\times 180$ .

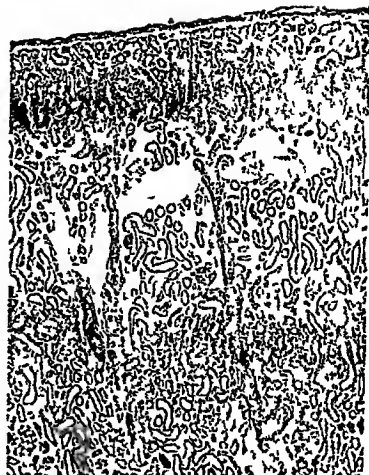


FIG. 11—54 hours. Kidney showing necrosis of the peripheral cortex, with leucocytic zones abundant below the ischemic area. H. & E.  $\times 50$ .



FIG. 12—Adrenal medulla showing the amount of adrenalin in the normal rabbit. Stained by Ogata and Ogata's method.  $\times 80$ .

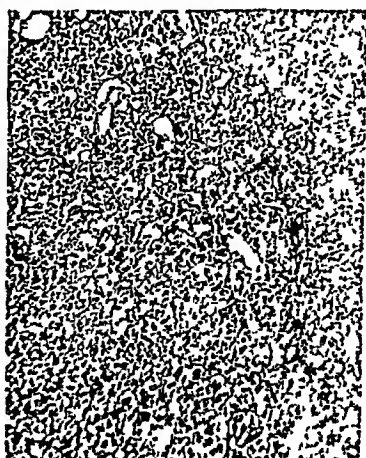


FIG. 13—Adrenal medulla of a rabbit injected with toxin showing the marked diminution in the amount of adrenalin. Stained by Ogata and Ogata's method.  $\times 80$ .





times showed an excess of clear fluid, but oedema of the cellular tissues was absent

### *Anomalous cases*

There was a group of animals, five in all, which showed functional changes such as hyperglycaemia, nitrogen retention, oliguria and albuminuria and which were expected to develop renal lesions. These changes, however, appeared to be transitory, and at autopsy there was no evidence of structural renal disease. An attempt to interpret these anomalies is made in the next section.

## PART II CIRCULATORY AND BIOCHEMICAL CHANGES RESULTING FROM INJECTION OF STAPHYLOCOCCUS TOXIN

For a proper understanding of the pathogenesis of the renal lesions following injection of staphylococcus toxin, it soon became clear that functional rather than morphological investigations would be required to elucidate the earlier stages. Certain of the functional disorders investigated, such as alterations in the rate of urine production and in the rate of elimination of phenol red from the circulation, appeared to be essentially renal in origin. Other disorders, however, seemed to originate outside the kidneys, and of these the most important, in view of certain current hypotheses on the pathogenesis of the related human condition, seemed to be an excessive release of adrenalin with resulting changes in arterial blood pressure and blood sugar. A careful comparison of the times of onset of these measurable functional changes with the times of development of the earlier structural changes described in part I is an essential preliminary to an understanding of the pathogenesis of the condition.

### *1 Changes in arterial blood pressure following injection of staphylococcus toxin in normal and adrenalectomised rabbits*

The systolic blood pressure, when measured by Grant and Rothschild's method in the central artery of the denervated rabbit's ear, varied considerably from one animal to another, but remained fairly constant in any individual animal under the same conditions of warmth and rest. The normal blood pressure in the denervated ear varied between 48 and 82 mm Hg in different animals, a wide variation which may have been due to the rabbits being mostly young and of different breeds and sexes. Differences in the size of the central artery may also have had some effect.

So far as the pathogenesis of the renal lesions is concerned, the only blood pressure changes needing consideration are those taking place prior to the onset of medial necrosis in the small arteries, i.e. in the 5 or 6 hours following injection of toxin. Of five animals,

normal except for ear denervation, whose arterial pressure was measured every 5 or 10 minutes after injection, one (no. 56) was found to have developed typical cortical necrosis when killed 50 hours later. The initial pressure of 48 mm. fell to 35 mm. five minutes after the injection, rose to a maximum of 61 mm. thirty-five minutes later, and returned to its initial level of 48 mm. after three hours. Between 3 and 50 hours after the injection, variations of a few mm. only were recorded. The four remaining rabbits (nos. 64, 65, 66 and 67) were killed before cortical necrosis had had time to develop. Though their glomeruli showed the characteristic histological picture of capillary congestion, their arterial pressures, as can be seen from table I, showed even less variation than did no. 56.

TABLE I

*Effects of injection of staphylococcus toxin in five normal rabbits.*

Rabbit no.	Interval before death	B P N (mm)	B P Min (mm)	B P Max (mm)	Histology	P R (per cent)	Fe (mg)
66	55 mins	79	71	76	Glom. congestion	8	0.99
64	2 hrs. 5 mins	74	62	69	" "	44	0.70
67	2 hrs. 40 mins	72	64	68	" "	7	0.74
65	2 hrs. 45 mins	82	55	92	" "	11	0.84
56	50 hrs	48	35	61	Cortical necrosis	5	.

B P = blood pressure N = before injection Min = minimum pressure after injection Max = maximum pressure after injection P R = phenol red excreted Fe = iron content per 1 g. of dried kidney

Five rabbits survived bilateral adrenalectomy sufficiently long for the action of the toxin to be investigated. One (no. 37), adrenalectomized one day beforehand, died 46 hours after the injection of 0.25 cc. of toxin per kg. with typical symmetrical cortical necrosis of the kidneys. There was persistent anuria following the toxin and the animal had a terminal blood urea of 333 mg. per cent. The arterial pressure, 62 mm. before adrenalectomy, fell to 45 mm. after that operation but before the injection of the toxin. The lowest pressure recorded during the five hours following the toxin injection was 41 mm. and there was no subsequent rise. After the first 24 hours it was impossible to obtain pressure measurements. Another rabbit (no. 61) died nearly 6 hours after injection and was found to show diffuse medial necrosis of its intralobular and afferent arterioles. Its arterial pressure after adrenalectomy but before injection of toxin was 60 mm., after injection it never fell below 56 mm. nor rose above 58 mm. Other particulars of this animal will be found in table II. An autopsy was performed immediately after death. The kidneys showed intense red mottling of the outer cortex, partly from

retention of phenol red, partly from vascular congestion. The bladder was empty. The adrenal sites showed no remnants of these glands, either grossly or microscopically. The three remaining adrenalectomised rabbits also showed no rise in arterial pressure following injection of toxin. Though they survived more than 24 hours, none developed cortical necrosis.

TABLE II  
*Protocol of rabbit no. 61*

Time	Blood pressure.	Blood sugar	Blood urea		
10 20 A M	60 mm	108 mg per cent	78 mg per cent		
The animal was catheterised and then 0.3 cc of toxin per kg + 1 cc phenol red were injected intravenously					
Time	B.P. (mm)	B.S. (mg)	B.U. (mg.)	P.R.	Urine excreted (cc)
10 25 A M	57				
10 45 "	58				
11 20 "	58	105		10 per cent	2
12 20 noon	57	74		Trace	0.2
1 30 P M	58	72	112		0.1
4 0 "	56		133	Nil	Nil
4 5 "	Death				

## 2 Changes in blood sugar following injection of staphylococcus toxin

Blood sugar curves were obtained on 30 rabbits which had been kept without food for the previous 24 hours. Blood samples were obtained just before and at hourly intervals after the injection of toxin. The resting blood sugar averaged 114 mg per cent with a standard error of  $\pm 3$  mg per cent. Ten control rabbits which received injections of inactivated toxin maintained their initial level, the mean of their estimations at the third hour being  $112 \pm 4^*$  mg per cent. Fifteen rabbits which received active toxin showed rises to a mean maximum of  $224 \pm 16^*$  mg per cent, the peak usually occurring between the 2nd and 3rd hour after the injection. In these rabbits a gradual return towards the initial level took place during the following 6-10 hours. This hyperglycaemic phase was constantly found after the injection of active toxin, and 12 of the 15 rabbits showing it died with fully developed cortical necrosis or the characteristic early changes which precede that condition.

In contrast to the findings of Delafield (1931) with Gram-negative bacteria, no second phase of hypoglycaemia was observed in any

\* The errors appended to the means are standard errors

rabbit On the contrary, the blood sugar level seldom returned completely to its initial figure and in some rabbits a secondary rise took place after 24 hours, concomitantly with an increase in the blood urea This secondary rise may possibly have been due to the accumulation of nitrogenous substances in the blood giving fictitiously high "sugar" figures (see Holden, 1926) Table III is the protocol of such a case

TABLE III

Protocol of rabbit no 15, black female		Weight 1900 g.
29/7/35		
11 A M	Resting blood sugar = 146 mg per cent 0.2 c.c. of toxin per kg intravenously	Urea = 41 mg per cent
12 A M	Blood sugar = 224 mg per cent	
1 P M	" " = 226 " "	
2 P M	" " = 216 " "	" = 46 " "
4 P M	" " = 186 " "	" = 70 " "
30/7/35		
11 A M	" " = 160 " "	" = 220 " "
4 P M	" " = 175 " "	" = 220 " "
5 P M	Killed The kidneys showed typical cortical necrosis	

Six rabbits (nos 30, 37, 42, 48, 50 and 61) on which the second adrenalectomy had been performed 1 day beforehand failed to show any rise in blood sugar after toxin They showed a mean figure at the third hour of  $104 \pm 6$  mg per cent, though later a small rise of 10 to 20 mg per cent was observed simultaneously with a rise in the blood urea The effect of adrenalectomy in abolishing the rise in blood sugar following injection of bacteria (*B. proteus* and *B. coli*) has previously been recorded by Evans and Zeckweil (1927)

These comparative observations on the changes in blood pressure and blood sugar and on the development of symmetrical cortical necrosis of the kidneys in rabbits following injection of staphylococcus toxin, show that adrenalectomy abolishes the two former, but does not prevent the occurrence of the third It seems clear, therefore, that the vascular reactions in cortical necrosis cannot be the results of either adrenalin release or of the production in the body of any adrenalin-like pressor substance

### 3 Disturbances of renal function following injection of staphylococcus toxin

These disturbances were indicated and measured by (i) alterations in the quantity of urine excreted, (ii) the presence of abnormal constituents in the urine, (iii) the excretion rate of phenol red, (iv) a rise in the blood urea concentration

(i) *Alterations in the quantity of urine excreted* The quantity of urine excreted by a normal rabbit varies from day to day but

under standard conditions of diet and room temperature is about 50 c c daily. Even under these conditions the output per hour varies too much from the average of about 2 c c to be a reliable index of changes taking place in renal function over short periods. The alterations in quantity after injection of toxin were, however, so great that they fell well outside the normal range. Twenty-three rabbits out of 37 showed either anuria or oliguria, 12 of them having empty bladders at autopsy 2-46 hours after injection. The 11 oliguric rabbits showed either a persistent or an intermittent reduction in the rate of urine excretion and the specific gravity of the urine formed by them was moderately high, 1016 to 1034, indicating fair concentration. Nine rabbits had some diuresis after injection of toxin. Though the kidneys of these animals subsequently showed cortical necrosis, the specific gravity of the urine produced during the period of diuresis was not markedly reduced, indicating that the concentrating power of the residual un-necrosed cortex was well maintained.

(ii) *Presence of abnormal constituents in the urine* Sugar and albumin were usually present in such urine as was formed during the first 12 hours after injection. Urine formed later was usually sugar-free but continued to contain albumin in small amount. Red cells and leucocytes were only occasionally present in the centrifuged deposits.

(iii) *The excretion rate of phenol red* This was done on male rabbits only, since they can be catheterised much more readily than females. The test was performed by the intravenous injection of 1 c c of a 0.6 per cent solution of phenol red, and was usually carried out on the rabbit both in its normal state and after injection of the toxin, the former serving as an individual control on the latter. The bladder was emptied by catheter 1 hour and 2 hours after the injection of the dye and the amount excreted in 2 hours expressed as a percentage of the amount originally injected. For purposes of comparison, the 2-hour percentage figure was preferred, partly because phenol red is excreted more slowly in the rabbit than in man and seldom appears in their urine, even under normal conditions, under 30-40 minutes, partly because over the longer period any divergencies from the normal become more apparent.

Eight normal rabbits excreted between 64 and 96 per cent of the injected dye in 2 hours, with a mean of 82 per cent. In 15 toxin-injected rabbits which received a simultaneous injection of dye, the amount varied from nil in those which became anuric to 44 per cent in those with diuresis, with a mean for the whole group of 15 per cent. At autopsy the peripheral part of the cortex of these rabbits was deeply stained bright red, in a diffuse or focal manner according to the distribution of the necrosis, but the inner

cortico-medullary zone always remained unstained. The medulla and pelvis were also unstained. Unfortunately it was not found possible, even by the use of alkalinised frozen sections, to determine at what level in the affected nephron the dye was being retained.

(iv) *The rise in the blood urea concentration* Though the rise in blood urea concentration is a late manifestation of the injuries produced by the action of the toxin, and indeed only becomes significant long after the first morphological changes are apparent, its determination provides a good index of the extent of the renal lesion. The blood urea concentration in normal rabbits varies between 25 and 55 mg per cent and may rise as high as 70 mg. per cent after 18 hours' fasting. In the adequately fed rabbit, it usually lies between 25 and 40 mg. per cent.

In rabbits injected with staphylococcus toxin, the blood urea remained within normal limits for the first 12 hours, thereafter rising with varying rapidity in different animals to reach figures of 200-300 mg per cent in 24 hours, and even to figures as high as 600 mg per cent. after 3 or 4 days. Such a rapid increase could hardly be due solely to nitrogen retention resulting from the injury to the kidneys: some animals had a blood concentration of over 400 mg per cent in spite of passing as much as 20 c.c. of urine in 24 hours, with a specific gravity of 1021 and a urea concentration of 2 per cent. Several other factors were probably concerned. Indeed it has already been stated that even after only 48 hours' fasting the concentration was raised appreciably, and, since a number of the toxin-injected rabbits were kept fasting for the first two days of the experiment in order not to interfere with the blood sugar curves which were being obtained at the same time, it is probable that part of the rise was due to fasting with its accompanying increased katabolism. In other rabbits, in spite of a full diet and notwithstanding the anuria with water retention, rapid loss of weight took place. In some instances this was as much as 100 g. a day, which, in a rabbit originally 2 kg. in weight, must have implied greatly increased tissue breakdown with corresponding increase in nitrogenous end products in the blood. Furthermore it was noted that after 24 hours it was often difficult to obtain arterial blood pressure readings. The ear arteries remained contracted even after well warming the rabbits suggesting a degree of circulatory failure that might well be responsible for part of the rise in blood urea by reducing blood flow in the unaffected part of the vasculature. It is probably fair to assume, therefore, that the rapid rise in the blood urea concentration following injection of toxin is due to several factors though the renal injury itself is probably the most important.

#### 4 *The iron and water content of the kidneys following injection of staphylococcus toxin*

Since the pathogenesis of symmetrical cortical necrosis appears to be essentially vascular, it is important to obtain as much information as possible on the reactions of the renal arteries between the time of the injection of toxin and the first appearance of parenchymatous changes in the nephrons. Previous studies on the blood pressure and blood sugar changes following administration of toxin, both in normal and adrenalectomised rabbits, had shown that the release of adrenalin or of an adrenalin-like pressor substance is not an essential precursor of cortical necrosis. On the other hand the histological observations on the condition of these vessels shortly after toxin had been injected suggested that, far from being constricted, the arteries of the intralobular and afferent types were widely dilated.

For the determination of the time of onset and the measure of development of this vascular dilatation, oncometry was contraindicated for two reasons. Firstly it entails severe operative procedures, and secondly it affords no means of distinguishing between changes in kidney volume due to the amount of urine present in its tubules and pelvis and those of vascular origin. It was therefore decided to attempt to gauge the vascular changes indirectly by determining the iron content of the kidneys of a series of rabbits killed at various times after the injection of toxin. This was considered reliable if the circulation was "fixed" by killing the animal rapidly and without struggling, and immediately clamping the renal pedicles prior to ligating, though it must be remembered that when comparing the normal with the abnormal values set out below, a certain percentage of each is derived from the renal cells themselves and not from the haemoglobin of the blood present in the organ. Murr and Shaw Dunn (1914-15) estimated the iron content of the perfused blood-free kidney in five normal rabbits and found that it averaged 0.27 mg per g of dried tissue. The kidneys of rabbits so killed were removed without loss of blood and weighed. The left kidney was dried at 105° C for 12 hours and then ground to a fine powder. The iron content of this powder was determined by Fowweather's method. The moisture content could also be determined. In order to obtain normal figures, determinations were made on 12 normal rabbits. The mean iron content per g of dried kidney was found to be 0.45 mg with a standard error of 0.013 and the mean moisture content 78 per cent with a standard error of 0.45 per cent. Eighteen rabbits were killed at intervals varying from 15 minutes to 20 hours after injection of toxin. Its injurious effects were estimated from the excretion rate for phenol red and



from later histological examination of the right kidney which was not needed for the iron estimation. All the animals showed a marked reduction in the percentage of phenol red excreted and animals allowed to survive for some hours showed the histological changes of cortical necrosis.

An increase in the iron content of the kidneys seemed to become evident about half-an-hour after the injection of the toxin, and at no time between 15 minutes and 20 hours was it observed to be below normal limits. Corresponding with the increase in iron content, there was also an increase in moisture. The findings are set out in table IV.

TABLE IV.

*Iron and moisture content of the kidneys in the normal rabbit and after the injection of staphylococcus toxin.*

Group	No. of animals	Time after injection	Average weight of the two kidneys as percentage of body weight	Average iron content (mg. per g. dry wt.)	Moisture content (per cent.)
1	12	normal	0.58	0.45	78.2
2	7	0 to 2 hrs.	0.75	0.61	80.8
3	7	2 .. 3 ..	0.92	0.72	82.1
4	6	3 .. 20 ..	0.92	0.74	83.3

The results of these iron determinations strongly support the views previously advanced from the histological evidence, that quite early, probably within less than half-an-hour, the renal vessels are in a state of dilatation. From the large number of investigations (Karsner and Austin, 1911) which have been made upon the period of survival of component renal cells, it becomes clear that, even were a temporary phase of vaso-constriction present, its duration would be much too short to produce the extensive parenchymatous lesions characteristic of the staphylococcus-toxin-produced cortical necrosis.

### DISCUSSION

The literature of symmetrical cortical necrosis of the kidney in man contains three main hypotheses sponsored by different authors. All have for their basis thrombosis of the affected renal arteries, the parenchymatous necrosis being supposedly secondary to the resulting ischaemia.

Jordan and Teacher (1910-11) thought that the thrombosis was caused by the spasmodic contraction of the renal vessels. S. de Navasquez (1930) thought that the state of abnormal vasomotor



by the fact that they fail to occur in adrenalectomised rabbits, which later often show quite characteristic vascular and parenchymatous necrosis. The adrenalin liberated appears to be responsible for the small rise in blood pressure, and there is every reason to believe that renal vaso-constriction takes place as part of the more generalised effect, but the constriction is only temporary and is not an essential precursor of the necrosis of the arterial media. These experiments indicate that toxin does not exert its effect in the arterial wall by way of release of adrenalin.

Recalling the three hypotheses outlined above, the possibility that the renal arteries enter into a prolonged state of spasm from some other vaso-constrictor than adrenalin has been examined by determining the iron content and presumably therefore the blood content of the kidneys at various times after injection of toxin. The results seem to show that no arterial spasm exists sufficiently long to bring about ischaemic necrosis of the renal parenchyma. Indeed, the iron content of the kidneys rises steadily in the first few hours after the injection of toxin, a result which can only be interpreted as resulting from an increase in the haemoglobin present. How an ischaemic necrosis of the cortex could develop under such conditions of apparent dilatation of the renal vessels, seems difficult to comprehend, but the following hypothesis is suggested as the most probable explanation.

After carrying out certain experiments on the rate of production of glomerular fluid with changes in the rate of blood flow, Winton (1932) came to the following conclusions: "The resistance to blood flow through the isolated kidney increases with arterial pressure. This is a unique property of the kidney, for other organs show a diminished resistance to blood flow at higher pressures. This increased resistance of the kidney is probably not due to vaso-constriction because in Ringer-perfusion the resistance to flow decreases with increased pressure as in other organs. The theory of glomerular filtration involves a removal of water from the blood in the glomeruli, and consequently the production of abnormally viscid blood in the vasa efferentia . . ."

The loss of water from the plasma as the blood passes through the glomeruli has been estimated by Mayrs and Watt (1922) for rabbits as varying from 6 to 36 per cent (average 19 per cent) of its volume, though most of the water is returned to the plasma subsequently by reabsorption through the tubules. When, as after injection of staphylococcus toxin, the afferent and intralobular arteries are paralysed prior to their necrosis, the glomerular capillary blood pressure must be raised to a level even higher than its usual high one, and the loss of fluid from the plasma must presumably be correspondingly increased (see Landis, 1927, p. 139). Such a loss of plasma fluid, with consequent corresponding concentration

of red corpuscles, must, as Winton has suggested, lead to an increase in the viscosity of the blood in the glomeruli and afferent arterioles. The observations of Trevan (1918) and of Whittaker and Winton (1933) show that the viscosity of blood increases disproportionately rapidly when the hæmatocrit exceeds 50 per cent.

The effect of this raised viscosity of the blood in the glomeruli and efferent arterioles, together with the raised blood pressure in the afferent arterioles resulting from the paralysis of the intralobular arteries, is to raise still further the blood pressure in the glomerular capillaries and increase correspondingly the loss of fluid from the plasma. A vicious circle thus develops which results finally in complete stasis of blood in the glomeruli and their afferent vessels as a result of packing of the red corpuscles.

Direct evidence in favour of this hypothesis can be obtained from the histological appearance of the kidneys in the early stages of the lesion. The extreme dilatation of the afferent arteries and of the glomerular capillaries, which are stuffed with masses of red corpuscles without intervening plasma spaces, is a constant finding. The severity of the glomerular distension is such as to obliterate most of the capsular spaces.

The early stages of the renal necrosis may thus be reconstructed. The intravenous injection of toxin leads to injury and paralysis of the intralobular arteries and afferent arterioles of the glomerular capillaries through which plasma fluid is rapidly lost, causing concentration of red corpuscles and consequent obstructive stasis. This stasis persists sufficiently long to bring about the ischæmic necrosis of the renal parenchyma.

The initial injury to the arterial wall may involve a greater or lesser part of the renal cortex, the ensuing anuria, oliguria or polyuria depending on the extent of the arterial injury and the number of nephrons affected. Further, the injury may not always proceed to medial necrosis, since a number of rabbits have shown all the characteristic early features of cortical necrosis such as oliguria, hyperglycæmia and raised blood urea for two or three days and have then recovered. The lack of histological evidence of renal necrosis in such rabbits suggests that the medial injury is readily recoverable in its slighter forms.

That staphylococcus toxin has a selective action on the renal arteries of rabbits in no way suggests that the same toxin causes the symmetrical cortical necrosis in human beings, though it is suggested that some agent with a similar vaso-selective action may be present in pregnancy or in those rare cases in which cortical necrosis arises in the course of some specific fever.

## SUMMARY

Intravenous injection of sublethal doses of *Staphylococcus aureus* toxin containing a hæmolysin produces in rabbits symmetrical cortical necrosis of the kidneys. The toxin causes a slight fall in blood pressure followed by a rise, which is accompanied by hyperglycæmia and glycosuria. The rise in blood pressure, hyperglycæmia and glycosuria are due to effects on the suprarenals and are abolished by adrenalectomy. This operation, however, does not prevent the development of renal necrosis after the subsequent administration of toxin. Areas of necrosis in the suprarenal cortex have been described in a number of animals. The pathogenesis of renal cortical necrosis is discussed in the light of the histological findings and functional changes.

## CONCLUSIONS.

1. The priority of the vascular lesion in symmetrical cortical necrosis of the kidneys has been established by experiment, confirming previous histological findings.

2. The vascular lesion consists of an acute necrosis of the media of the intralobular and afferent arteries of the peripheral renal cortex and is unaccompanied by thrombosis.

3. The medial necrosis is preceded by dilatation of the afferent arterioles and glomerular capillaries. There is no evidence that vaso-constriction or spasm occurs.

4. The vaso-dilatation is the result of loss of muscular control caused by paralysis of the affected arteries, and leads to obstruction of the glomerular circulation. The mechanism of the obstruction is thought to be the rapid loss of plasma through the grossly dilated glomerular capillaries due to heightened pressure in the renal artery. The resulting concentration of red corpuscles leads to circulatory stasis with consequent ischaemic necrosis of the parenchyma.

It is a great pleasure to express my gratitude to Professor G. Payling Wright for his constant help and stimulating advice. I am also indebted to Dr H. J. Parish of the Wellcome Physiological Laboratories for generous supply of staphylococcus toxin, and to Dr R. T. Grant for his kindness in determining his apparatus for measuring the blood pressure in rabbits.

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## THE URINARY EXCRETION OF PNEUMOCOCCUS POLYSACCHARIDE IN LOBAR PNEUMONIA

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IN 1917 Dochez and Avery showed that types I, II and III pneumococci produced a "specific soluble substance" (SSS) during the active phase of growth in nutrient broth. They found a similar substance in the serum and urine of experimentally infected rabbits and in the urine, less frequently in the serum, of patients with lobar pneumonia due to these three common pneumococcal types. The specific substances have since been identified as polysaccharides and are present in the capsule surrounding the pneumococcus cell. Although the detection of specific substance in the urine has recently been recommended, and probably used, as a method for the serological typing of pneumococcal infections, there has not apparently been any published confirmation of Dochez and Avery's findings, and the present investigation was begun in order to determine the diagnostic and prognostic value of the test in patients with lobar pneumonia, with special reference to serum therapy. As the work progressed, it was noted that excretion of the specific substance was commonly continued during convalescence and a discussion of this phenomenon is included in the paper.

### *Material and methods*

The specimens were obtained from male patients between the ages of 15 and 65 admitted to the pneumonia wards of Belvidere Isolation Hospital, Glasgow. The present analysis concerns material from 200 patients with pneumococcal infections, of whom 184 were suffering from lobar pneumonia (type I, 44; type II, 103; type III, 7; group IV, 30), while in 16 the diagnosis was bronchopneumonia or bronchitis. Over a thousand specimens of urine were examined. The test is a precipitation reaction, the specific substance as antigen or precipitinogen being detected by mixing small volumes of the urine with suitable dilutions each of types I, II and III pneumococcal antisera of good precipitating action. With urine as the diluent of 1 per cent solutions of the three purified polysaccharides and the sera diluted 1:5 with normal saline, the type I antiserum gave a positive precipitation reaction with 1:1,000,000 dilution of the corresponding SSS, type II with 1:2,000,000 and type III with 1:500,000. However, the



polysaccharides used were degraded preparations and recent observations have shown that the whole or undegraded pneumococcus polysaccharides react in higher dilution than do those prepared by the older methods. As the polysaccharides appear in the urine in undegraded form (Avery and Goebel, 1933) it is reasonable to suppose that a dilution of at least 1 5,000,000 of the types I and II specific substances would be detected with the antisera used in the present investigation.

A morning specimen (about 10 ml) of urine was sent to the laboratory on the day after the patient's admission and, in many cases, further specimens were sent at two- or three-day intervals until dismissal or death of the patient. Specimens of sputum for serological typing of the infecting pneumococcus by the direct and mouse-inoculation methods were similarly sent. Not all samples of urine or sputum were examined and specimens of urine were discontinued from patients suffering from group IV pneumococcal pneumonia except in a group of 30 whose urines were repeatedly examined throughout the acute and convalescent stages to test the specificity of the precipitation reaction. At first the urinary specimens were examined for specific substance without any preliminary treatment, but as many of the tests showed non-specific deposits of phosphates the results were discarded: the pH of all further specimens of urine was adjusted to 8.0 with N/1 NaOH and the phosphates were sedimented by centrifuging or allowing the specimen to stand on the bench for 2 hours.

The test consisted in placing 0.3 ml of the clear supernatant urine into each of three Dreyer's agglutination tubes and layering on top of the urine in tube 1, 0.2 ml of type I pneumococcal antiserum diluted 1/5 in normal saline, in tube 2 a similar amount of type II antiserum and in tube 3 type III antiserum. Two readings were taken. (1) the tubes were immediately examined under optimal lighting for the development of a white ring at the junction of the two fluids. (2) after standing on the bench at room temperature for 4 hours, the presence and amount of deposit was noted and recorded as +, ++ or ++++. Although there was close agreement between the two readings, the presence of a specific deposit after standing was regarded as the more reliable and no test was recorded as positive without it. There was apparently no acceleration of the reaction by incubation at 37° C or placing in the water-bath at 56° C. While at first all specimens of urine were tested with types I, II and III pneumococcal antisera, later, when the specificity of the reaction was proved, many urines were tested only with the appropriate serum. For example, if a case were shown to be a type II pneumococcal pneumonia by examination of the sputum and a specifically positive urinary precipitation test were obtained with type II antiserum, subsequent specimens of urine from that patient might be tested only with the type II serum.

*The specificity of the reaction and amount of polysaccharide excreted.*

Obviously if the test is to be of diagnostic value for determining the serological type of the infecting pneumococcus, it must be strictly specific. About half of all the specimens of urine were tested with all three anti-pneumococcal sera. These included repeated samples from 30 cases of group IV pneumococcal pneumonia and from 16 patients with bronchitis or bronchopneumonia, while with each batch of tests urine from a healthy person was included as a control. The results showed an almost complete agreement

in the serological type of the infecting pneumococcus as determined by examination of the sputum and the urinary precipitation test when the latter was positive. In only one instance was there persistent disagreement between the type of pneumococcus isolated from the sputum (group IV) and the urinary precipitation test (type I) and here the clinical history of the case suggested that the urinary finding was the correct one.

Attempts were made to estimate quantitatively the amount of specific polysaccharide excreted daily in the urine, although this could only be done accurately by sampling from a measured 24 hours' collection. At first the optimal proportions method of Dean and Webb (1926) was tried but satisfactory results were not obtained with urinary specimens, probably because of the high concentration of salts present. Instead, precipitation tests were done with increasing tenfold dilutions of urine (1:10 to 1:10,000) in a series of 20 positive specimens. Positive results were usually obtained in dilutions of 1:10 or 1:100 which, allowing detection of the polysaccharide in a dilution of 1:5,000,000, would represent a concentration of 0.0002 to 0.002 g per 100 ml or, with a calculated daily output of 1500 ml of urine, a daily excretion of 0.003 to 0.03 g of the specific polysaccharide.

*Serological typing of pneumococcal infections by the  
urinary precipitation test*

The demonstration of specific polysaccharide in the urine early in the infection would be of great value for the serological typing and serum treatment of lobar pneumonia since the test is so easily and quickly carried out. Unfortunately, the reaction is positive only in a proportion of cases—62 per cent in the present series of 154 cases of types I, II and III pneumococcal pneumonia, which is in agreement with Dochez and Avery's figure of 65 per cent (52 out of 80) with concentrated specimens of urine (table I). The proportion of positive findings is significantly higher in type II (74 out of 103 or 71.8 per cent) than in type I infections (20 out of 44 or 45.5 per cent), so that the total percentage positive would be lower in a series of cases where type I was the commoner infecting type. Another drawback to the method is the fact that the first or second specimen examined may be negative and the test may only become positive late in the infection or even after the crisis. For example, of the types I, II and III infections together, the reaction was positive with the first sample of urine in 41.5 per cent of cases in the type I pneumonias it was positive in 9 out of 44 or 20.5 per cent and in the type II cases in 53 out of 103 or 51.5 per cent, so that the precipitation test would be useful for early diagnosis in about one-half of type II pneumococcal

pneumonias and in one-fifth only of type I infections. The complement fixation test using *rabbit* instead of horse antiserum detects the presence of specific polysaccharide in a four- or five-fold greater dilution than does the precipitation test (Downie, 1937a) and I am indebted to Dr A. W. Downie for testing 6 samples of urine, which gave negative precipitation tests, from two patients with type I pneumococcal pneumonia. However, with these samples the complement fixation tests were also negative, so that besides the greater technical difficulties of this method, it would probably not give an appreciably higher percentage of positive results than the precipitation reaction.

TABLE I.  
*Diagnostic value of the urinary precipitation test.*

Type	Present investigation			Dochez and Avery		
	No. of cases	No. positive	Percentage positive	No. of cases	No. positive	Percentage positive
I	44	20	45.5	35	20	57.1
II	103	74	71.8	28	20	71.4
III	7	2		17	12	70.5
Total	154	96	62.3	80	52	65.0
Group IV	30	0	0	10	0	0
Miscellaneous	16	0	0	14	0	0

1st specimen positive (types I, II and III), 64 or 41.5 per cent (I, 0 or 20.5 per cent, II, 53 or 51.5 per cent)

Despite its limitations in the early diagnosis of serological type, it is obviously a useful confirmatory test, giving a higher proportion of positive results than blood culture and being so much more easily and quickly performed than either blood culture or lung puncture. In certain instances, usually where there was great difficulty in obtaining a specimen of sputum, it established the type diagnosis, while in several cases it has confirmed a clinical diagnosis of reinfection occurring in the wards. Pleural fluids and other exudates suspected of being pneumococcal in origin have also been tested for the specific polysaccharide and with such material the test will give a rapid diagnosis or detect the infecting type when the causal organism cannot be recovered.

*The prognostic value of the urinary precipitation test*

The presence or absence of pneumococcal polysaccharide in the urine deserves a place among the prognostic signs in lobar pneumonia, especially as a guide to serum therapy. Of the total

types I and II pneumococcal pneumonias with positive urinary precipitation tests 25 out of 94 or 26.6 per cent died, whereas only 1 died out of 53 patients (1.9 per cent) with negative tests, and in this instance only one specimen of urine was examined (table II). It may be said therefore that a good prognosis should

TABLE II  
*Prognostic value of the urinary precipitation test*

Type	No positive	No died	Percentage died	No negative	No died	Percentage died
I and II	94	25	26.6	53	1	1.9
I	20	4	20.0	24	0	0
II	74	21	28.3	39	1	3.4

1st specimen positive, 02, died, 24 (38.7 per cent)

be given if persistently negative results are obtained during the four or five days preceding the expected critical fall of temperature. As for positive reactions, the prognostic value of the test is probably enhanced if only the first specimen be taken as a guide (since in the present series 24 out of 62 or 38.7 per cent of patients died whose first urinary specimen contained pneumococcus polysaccharide), although the fact cannot be regarded as statistically established on the data available. The prognosis is especially grave when the result of the first test is recorded as +++ or where the precipitate increases with successive specimens from + to +++. In view of these results, it seems reasonable to recommend serum therapy for patients with type I or II pneumococcal pneumonia whose early urinary specimens give a positive precipitation test.

Two of the most important factors influencing the mortality rate in lobar pneumonia are the age of the patient and the type of infecting pneumococcus and it is interesting to examine the relationship of the urinary precipitation test to these two prognostic factors (table III).

TABLE III  
*Urinary excretion of SSS in relation to age and type*

	No of cases (types I and II)	No died	Percentage died	Percentage SSS positive
Age—				
15-31	92 (28+64)	12	13.0	59.6
35-65	55 (16+39)	14	25.5	72.7
Type—				
I	44	4	9.1	45.5
II	107	22	21.3	71.8

*Age of patient* If the types I and II pneumococcal pneumonias are divided into two age groups, 15-34 years and 35-65 years, the crude mortality rate in the first group is 13 per cent and the incidence of positive reactions 58·6 per cent, while in the older age group the corresponding figures are 25·4 per cent and 72·7 per cent. Approximately 30 per cent of the cases in each age group were type I infections. There is thus a rough parallelism between the death-rate and the precipitation reaction in the two age periods, the higher mortality in the older group being reflected in a higher percentage of positive reactions. The death-rate of patients under 35 years of age with demonstrable pneumococcus polysaccharide in the urine was 22 per cent (12 out of 54) and in the group over 35 years, 35 per cent (14 out of 40) which suggests that a positive precipitation test has a more serious prognostic significance in older patients, though the data are not adequate to establish such an inference.

*Type of pneumococcus* Reference has already been made to the higher percentage of positive results in type II than in type I infections. When the death-rate from lobar pneumonia due to these two types is correlated with the incidence of urinary excretion of the corresponding polysaccharides, there is again a fair degree of parallelism. The figures for type I infections are, death-rate, 9·1 per cent, positive precipitation tests, 45·4 per cent, and for type II, 21·3 per cent and 71·8 per cent. An explanation of the difference in death-rate and in symptomatology between type I and type II pneumococcal pneumonias has previously been suggested (Cruickshank, 1933) and the present finding of a higher proportion of positive urinary precipitation tests in the severer type II infections is consistent with the view there expressed that the greater toxicity of the latter is associated with a greater quantitative elaboration in the tissues of the soluble specific substance.

*The urinary excretion of pneumococcus polysaccharide  
during convalescence*

Specimens of urine from 91 patients who recovered from type I or II pneumococcal pneumonia were examined at two- or three-day intervals from the time of admission to hospital until discharge. Specific polysaccharide was present in the urine of 54 cases: in 10 (18·5 per cent) the test was negative before the crisis or lysis and became positive in convalescence, while in only 7 patients (12·9 per cent) was it positive before and negative after the fall of temperature. Thus of 54 patients in whose urine specific pneumococcal carbohydrate was demonstrated, 47 or 87 per cent were excreting the substance during convalescence. In fact most

of the patients—and especially those with type II infection—continued to excrete SSS as long as they were in hospital, a period normally of 3 to 4 weeks (see fig). It was thought possible that this continued excretion was associated with a continuance of the infective process, either as a delayed resolution of the pneumonic consolidation or as a serous or purulent pleural effusion, since in 7 patients with a protracted stay in hospital because of such complications the average duration of polysaccharide excretion was 43 days

	CASE	AGE	DAYS																	RESULT
			6	3	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	
TYPE I	JG	28	-	■	■		■	■	■											WELL
	AMN	45					■	■	■											WELL
	AMPW	52		■	■	■														DIED
	JR	36		■	■		-	-	-	-	-	-	-	-	-	-	-	-	-	WELL
TYPE II	SK	56	■	■		■	-			■	■		■	■	■					WELL (DEL. RESOLUTION)
	KS	26		-	-	■	■	■	■	■	■	■	■	■	■	■	■	■	■	WELL (PLEURAL EFFUSION)
	RH	42	■	■	■		-	-	■				■							WELL
	GMD	61		■	-	■	■	■	■							■				WELL
	NH	45	■	■	■	■	■	■	■	■			■	■				-	-	WELL (DELAYED RESOLUTION)
	JL	30	■	■	■															DIED

■ PRECIPITATE +  
 ■ ++  
 ■ +++  
 - - - - -

FIG.—Examples of the urinary excretion of pneumococcus polysaccharide in types I and II lobar pneumonia

(34-55 days) But further analysis showed that certain cases with delayed resolution ceased to excrete the substance before dismissal while three patients with type I pneumococcal empyema never at any time had demonstrable SSS in the urine. Again, cases without complication continued to excrete specific polysaccharide for 20-30 days and two patients discharged well still gave positive reactions when they returned for re-examination on the 34th and 39th days respectively of their convalescence. Another possibility was that the pneumococcal autolysis which

presumably goes on in the lung tissue at or about the time of the crisis was responsible for the excretion of the specific substance after the fall of temperature. But certain patients with quite extensive consolidation had not at any time detectable polysaccharide in the urine and in a small number of cases the test was positive before and negative after the crisis. In others the amount of precipitate formed seemed to diminish about the time of the crisis only to increase again later in convalescence.

What then is the explanation of the continued excretion of the pneumococcus specific substance after the infection has been overcome? The polysaccharides behave, in mice and men, like true antigens and, while not to be regarded as toxins in the limited sense of the word, they undoubtedly play an important part in pneumococcal infections. Although Dochez and Avery found S S S in the serum of only 8 out of 25 patients with lobar pneumonia by the precipitation test and only 2 out of 8 sera examined by me gave positive results, Park (1931) has shown that a substance—presumably the polysaccharide—which specifically neutralises pneumococcal antiserum is present in appreciable amount in the blood of severe cases of lobar pneumonia. It was therefore to be expected that the polysaccharide if excretable should be present in the urine during the acute stage of the infection, although in fact it was demonstrable in not more than 56 per cent of the present series of cases during the fastigium.

After recovery, when specific pneumococcal antibodies are usually present in the blood, it is unlikely that pneumococcus polysaccharide would circulate as free antigen or hapten although in the present series 1 out of 8 sera obtained during early convalescence gave a positive precipitation reaction and Downie (1937a) has recently shown that demonstrable pneumococcus polysaccharide in the blood of mice is not incompatible with some degree of resistance to infection with the homologous organism. However, in the presence of detectable humoral antibodies—and these are present in the majority of recovered cases of lobar pneumonia—the homologous polysaccharide is very quickly cleared from the blood stream after intravenous injection (Downie, 1937b). Avery and Goebel found that type I pneumococcus polysaccharide when injected into normal rabbits is very slowly excreted, since it was present in the serum 7 days after injection and when given in large amounts (17 mg) could be demonstrated undegraded in the urine for at least 7 days. These established facts may afford an explanation of the continued excretion of polysaccharide in the urine of patients convalescent from lobar pneumonia. With the formation of antibody the circulating antigen becomes “fixed,” probably mostly by the tissue cells, but instead of being catabolised it may gradually become dissociated from the antibody and be

excreted by the kidneys. The slow and continuous excretion may be due partly to the slow rate of dissociation and partly to the difficulty of elimination because of the large size of the molecule. Its apparent absence from the serum when detectable in the urine is possibly explicable by the concentration which it undergoes in the process of excretion. Possibly the specific substances and toxins of other pathogenic bacteria are similarly dissociated and excreted during the patient's convalescence, although the data available on this point are surprisingly scanty.

### Summary

The specific polysaccharides of types I, II and III pneumococci are excreted in the urine of the majority of patients with lobar pneumonia due to these three types and may be demonstrated by a precipitation reaction. Thus urinary precipitation test has only a limited value as a method for the early serological typing of lobar pneumonia, as the first specimen of urine examined was positive in only one-fifth of the type I and one-half of the type II infections. The detection of the polysaccharide in the urine early in the infection has, however, a grave prognostic significance since the mortality in such patients was around 40 per cent whereas less than 2 per cent died whose urinary specimens were persistently negative. The pneumococcus specific substance continues to be excreted in the urine during the patient's convalescence and in explanation of this phenomenon it is suggested that the polysaccharide as antigen is gradually dissociated from the antibody and slowly excreted by the kidneys.

The work on which this paper is based was done in the Pathology Department of the University and Royal Infirmary, Glasgow. I am indebted to Dr J. A. Montgomery, Belvidere Hospital, for arranging for the collection of specimens and for the clinical data, and to Dr P. Hartley, Sir Patrick Laidlaw and Dr R. A. O'Brien for supplies of types I, II and III pneumococcal antisera and purified polysaccharides.

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# THE SOURCE OF THE FAT IN EXPERIMENTALLY PRODUCED FATTY DEGENERATION OF THE HEART

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(PLATES V AND VI)

IN a previous paper (Dible, 1934) we criticised the view that a process of phanerosis is responsible for the production of "fatty degeneration" of the heart in man, and adduced evidence that such a conception is unnecessary. Amongst the observations which have supported this theory are those of Leathes and Dudgeon (Leathes, 1906), who investigated the fat content and histological appearances in the hearts of guinea-pigs which had been poisoned by diphtheria toxin. Using a number of pooled hearts they found 19.77 per cent of fat in the myocardia of the poisoned animals, whereas in a series of normal animals the percentage of fat was 17.43. They assumed that the small difference of 2.34 per cent was insufficient to account for the marked fatty change which histological examination showed to be present in the hearts of the toxin-treated animals and concluded that there must be an unmasking of the unstainable fat of the muscle cells. The weights given refer to dried heart muscle: the equivalents for the fresh tissue were 2.23 and 2.05 per cent—a difference of about 0.2 per cent.

In our own investigations (Dible, 1934) upon the human heart we found that the cardiac muscle of individuals killed suddenly by accident, or dying speedily under conditions in which no pathological changes in the myocardium would be anticipated, contained about 1.8 per cent of fat with a range of 1.2–2.1 per cent, all of which was invisible (*élément constant*, or masked fat). In hearts showing the patchy form of fatty degeneration we found a higher content of fat in the portions of the ventricular muscle in which the naked eye change was well developed than in those portions in which such change was absent or minimal. If, for our present purposes of comparison, we take the mean of the findings in both fatty and non-fatty areas in such hearts, and also include those hearts in which the type of fatty change was diffuse, we find that in a total



smaller animals 0.23 cc, 1 cc for each group about 0.5-0.8 mg of P per 100 g of live body weight. Death in both series occurred between the 5th and 8th days, the average survival period being 6.2 days for the smaller and 6.8 days for the larger guinea pigs, one or two of the animals were killed when *in extremis*. The animals lost some 20-30 per cent of their gross weight, the amount lost depending mainly upon their length of survival, so that a definite element of starvation entered into these experiments. The factors of dosage and length of survival did not, within the limits of our experiments, appear to have a marked effect upon the production of the fatty changes.

After death the heart was freed from all visible fat, fibrous tissue and blood, and a small fragment of the ventricle was taken for histological examination. The organ was then weighed and the fat estimated by the saponification method. The liver was dealt with similarly. The contents of the alimentary canal were then removed and the carcass was weighed, this, added to the weights of heart and liver, gave the nett weight at death. The whole carcass was then saponified for the estimation of the total fat in the animal. This is expressed in the tables as a percentage of the nett weight.

### Results

In this investigation we are more especially concerned with the results in the heart, but since contemporaneous examinations were

TABLE I  
Control animals

No	Nett weight	Percentage of fat in		
		body	heart	liver
A Unfattened				
12	231	4 15	1 32	2 39
7	286	4 30	1 50	2 06
5	323	4 40	2 02	
2	410	4 83	2 07	2 07
0	303	6 70	1 39	2 52
Range	231 410	4 15 0 70	1 32 2 07	2 06 2 07
Mean	312	4 80	1 58	2 47
B Fattened				
1	775	6 88	1 96	2 72
3	551	7 00	1 78	2 16
10	652	10 90	1 73	2 32
8	770	11 10	1 70	2 70
0	918	14 74	2 12	2 77
Range	551 918	6 88 14 74	1 73 2 12	2 16 2 77
Mean	734	10 24	1 88	2 53

made upon the liver these are included in brief form for the sake of comparison and completeness. The results in 10 control animals

are given first and are followed by those in 16 animals which were poisoned with phosphorus. Two other animals in the control group were found to be pregnant and are excluded from the analyses, since fat metabolism tends to be disturbed in pregnancy. Actually their inclusion would not have affected the results. In all tables the animals are arranged in order of bodily adiposity.

TABLE II  
*Phosphorus-poisoned animals*

No	Nett weight	Weight lost in experiment	Percentage of fat in		
			body	heart	liver
C Unfattened					
P 4	304	41	1 54	*1 43	3 05
P 3	279	112	1 65	*1 43	3 50
P 11	265	114	2 12	*1 55	5 16
P 16	206	75	3 70	2 28	5 36
P 7	236	128	4 08	2 65	5 46
P 13	299	82	4 22	2 32	6 90
P 5	330	100	4 98	†1 84	7 00
P 10	290	59	5 88	1 94	11 12
Range	206-330		1 54-5 88	1 43-2 65	3 05-11 12
Mean	276		3 52	1 93	5 94
D Fattened					
P 2	677	262		*2 01	6 30
P 9	512	134	6 15	3 36	10 06
P 6	568	238	9 62	†1 61	4 17
P 15	697	150	11 74	2 43	13 75
P 14	633	150	12 90	2 35	7 72
P 1	744	106		2 74	10 07
P 8	730	109	14 02	2 32	12 14
P 12	730	133	15 04	2 21	13 30
Range	512-744		6 15-15 04	1 61-3 36	4 17-13 75
Mean	662		11 57	2 38	9 66

\* These animals did not show any histological fatty change in the heart muscle

† These animals showed only slight traces of histological fatty change

None of the animals of either control series showed any histologically demonstrable fat in the myocardium, and this was also absent in P 2, P 3, P 4 and P 11 of the experimental series, whilst P 6 and P 5 showed only slight traces of fatty change. The four former are therefore excluded from further consideration, since in them we have failed to produce fatty degeneration, but the latter are included although the amount of degeneration produced was minimal. When this correction has been made the mean

values in the different series are those given in table III and shown graphically in fig 1

TABLE III

	Percentage of fat in		
	body	heart	liver
<b>A Unfattened Animals</b>			
A Controls	4.89	1.68	2.47
C Phosphorus poisoned	4.57	2.20	7.17
<b>B Fattened Animals</b>			
B Controls	10.24	1.88	2.53
D Phosphorus poisoned	11.57	2.43	10.77

The mean increase in fat content of the heart muscle of the poisoned animals in each series over the corresponding controls is about 0.5 per cent

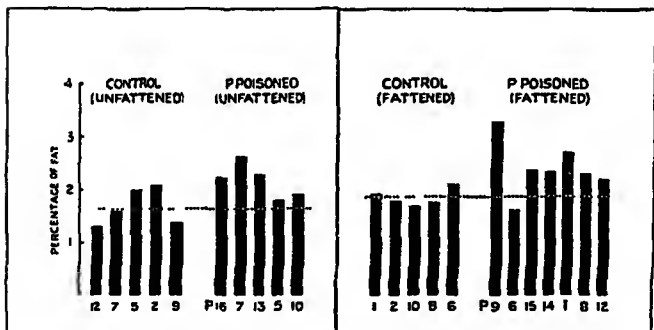


FIG 1—Amounts of fat found in the hearts of control guinea pigs and in those developing fatty degeneration as a result of phosphorus poisoning. The horizontal lines show the mean value for the control animals in each group (1.68 per cent for the unfattened and 1.88 per cent for the fattened animals)

Although these increases are not large they are significant in so far as small series such as these lend themselves to statistical analysis. For this purpose, to deal with the largest possible number of observations, we may take both control groups (A and B) together and also the relevant results in the two experimental series (C and D). If this be done the observed difference between the two means is 0.56 per cent, which is more than three times the standard

error of the difference of the means (0.158). What is perhaps of equal moment is that the differences between the fat content of the hearts of the poisoned and normal guinea-pigs in the general run of the animals as shown in tables II and III are very consistent throughout. The conclusions which we draw from these findings are as follows:

1. There is an increase in the fat content of the heart muscle where the visible changes of fatty degeneration are produced.

2. The fat content of the normal heart muscle may be higher in animals which are fattened than in those which are not, it is possible that this is due to some variation in the *élément constant* of the muscle in relation to the animal's nutrition.

3. In keeping with (2), the increase in extractable myocardial fat produced by phosphorus poisoning is of about the same order in both groups of experimental animals, but the absolute fat content is generally higher in the fattened than in the unfattened series.

It must be allowed that the experimental error in estimating the quantity of fat in a single guinea-pig's heart may be considerable, since the amount of tissue available is between 0.55 and 1.86 g., none the less, as we have pointed out, the values obtained in each class indicate that the results are consistent. A value of 2.0 per cent. and upwards is only found in three of the control animals, with a maximum of 2.1, whilst this value is exceeded in nine of the phosphorus-treated animals with a maximum of 3.4 per cent. All these animals showed definite histological "fatty degeneration."

The results also show the customary changes in the liver and the correlation between the quantity of fat in the storage depots and the degree to which the liver is infiltrated. This is well shown in the animals which have a moderate amount of storage fat (series C). All the phosphorus-poisoned animals showed some degree of visible fatty infiltration in the liver. This was variously distributed but in most cases there was a marked predilection for the portal zones. The size of fat globule seemed chiefly governed by the quantity of fat present, where this was small the globules tended to be small, where the increase was great, large globules of fat were produced.

*Histological correlation.* The histological preparations which showed visible myocardial fat were examined by three separate observers, each of whom arranged them in order of the severity of the visible change. While there was some disagreement about closely similar preparations, there was close general agreement between the order thus determined and that given by the chemical analyses. The histological picture in the hearts which showed the smaller quantities of fat was that of scattered, patchy and infrequent areas of change (animals P 5 and P 6, fig. 2) whilst in the hearts which showed a greater increase the change was diffuse (fig. 3). In most cases the fat globules were fine. The

## EXPERIMENTAL FATTY DEGENERATION



FIG. 2—Phosphorus poisoned animal P 5  
1.81 per cent of fat. Histologically only  
a few odd patches of fatty change, one  
being shown in the figure. Scharlach R  
 $\times 150$

FIG. 3—Phosphorus poisoned animal P 1  
2.74 per cent of fat. General fine  
peppering of the muscle fibres.  
Scharlach R  $\times 310$



FIG. 4—Phosphorus poisoned animal P 9  
3.36 per cent of fat with notable peri-  
vascular concentration. Scharlach R  
 $\times 90$





## EXPERIMENTAL FATTA DEGENERATION



FIG 5 — Phosphorus poisoned animal P 9 3 36 per cent of fat General fine peppering of the muscle fibres, with more intense perivascular change Scharlach R  $\times 200$

FIG 6 — Emulsion in agar containing 0 14 per cent of fat Scharlach R  $\times 280$

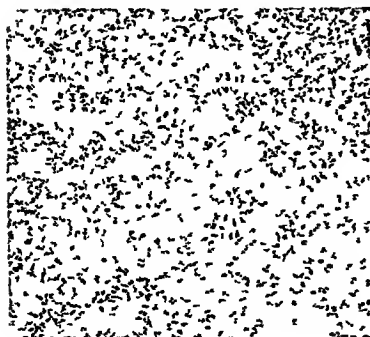
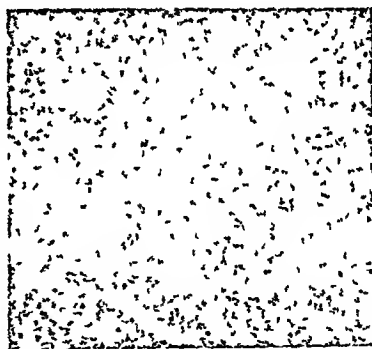


FIG 7 — Emulsion in agar containing 0 28 per cent of fat Scharlach R  $\times 280$



figures show typical examples of different grades of change. We would call attention to the marked perivascular distribution of the fat in one animal (P 9 figs 4 and 5), which is comparable to the periportal distribution so often seen in the liver as a result of the action of phosphorus.

### *Discussion*

In the first place it should be pointed out that certain of the poisoned animals failed to show any myocardial fatty degeneration (P 2, P 3, P 4 and P 11). These were the animals with the poorest fat reserves, so that here we find a repetition of the phenomenon so familiar in the liver—that fatty change is dependent upon adequate fat in the storage depots—the observation, in fact, which first directed Lebedev's attention to the importance of the infiltration mechanism in that organ. It is significant to find the same phenomenon in the heart.

The increases we have shown in our experiments are not large and it will be asked how much regard is to be paid to increases in fat content which on an average are of about 0.5 per cent. It is apparent from the general results of all workers that the heart muscle has not the capacity of the liver cell to accumulate fat, indeed it would be extremely difficult to visualise it performing its contractile function when thus laden, and it is exceptional in analyses to find many values of over 3 or 4 per cent. It is therefore misleading to compare liver and heart without allowing for this essential difference in the tissues. Small as these increases are they are nearly three times those recorded by Leathes and Dudgeon. Apart from differences in the technique of fat estimation and in the method of inducing fatty change there are two factors to be considered which bear upon the discrepancies between their results and ours. In the first place they pooled a number of hearts for their estimations whereas we have worked with single hearts, in the second place they did not control their experimental animals by estimations of the total fat in the animals' bodies, although they chose animals of approximately the same size. Failure to do this may lead to errors. If, for example, we were to use as controls for our unfattened experimental animals the normal animals of our fattened group, the mean increase of fat found in the hearts would be only 0.32 per cent, a figure not very much greater than theirs. In this connection we may point out that the mean fat content of the heart muscle in their controls was 2.05 per cent, a figure considerably in excess of that for either of our control series.

It is apt to be overlooked that the ordinary method of expressing the fat content of the heart muscle as a percentage wholly fails to give an accurate impression of the quantity of the change, since it relates the fat found to the whole of the heart muscle whereas very

often large areas of myocardium may be quite free from fatty change. The force of this point was clearly brought out in our work upon the human heart (Dible, 1934) in which we were able to contrast the fat content in the degenerated areas, which could be separated by dissection, with the fat content of the relatively healthy areas. As a result we were able to demonstrate increases in the fat content of the affected muscle of a much higher order than those obtained in these experiments. Consequently the actual increase in fat in the affected areas in these small hearts must be very much greater than the analyses show, except where the whole of the myocardium is uniformly affected.

Finally, any purely histological estimate of the amount of fat in a tissue can only be a very crude affair, partly by reasons of the irregular distribution of the fat and more particularly because personal impressions without adequate standards of reference are bound to be inexact. As a result of a good deal of work upon fatty change in different tissues we have long been of the opinion that relatively small quantities of fat can give quite an impressive picture when highly dispersed, which is the condition in which the fat globules are usually present in heart muscle. This opinion might be criticised as a mere begging of the question but for the fact that it has been reached largely from the study of sections of the liver in which all the evidence has pointed to the visible fat being a result of simple infiltration, and also that we have been able to obtain support for this view from artificial preparations.

Emulsions of fat in a high state of dispersion were made in agar and after cooling were fixed in formalin and osmium tetroxide. Both frozen and paraffin (formol- and osmic-acid-fixed) sections were cut. These give us a picture of fat emulsions of known content in which all the fat is stainable and equally diffused and no possible question of unmasking confuses the issue. Their study shows us that quantities of fat of the order of 0.1–0.3 g per cent give a striking visual picture (figs. 6 and 7), and one quite comparable with that seen in tissue in which the excess of stainable fat over the content in masked fat is of the same order.

### Conclusion.

From the study of the myocardium of guinea-pigs poisoned with phosphorus, using as controls animals in similar states of nutrition, we conclude that the fatty change which may be produced is of the nature of an infiltration and that there is no reason to assume the unmasking of the essential fat of the cell.

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# THE IMPORTANCE OF HYDROGEN ION CONCENTRATION IN HÆMOLYSIS BY THE LYSINS OF ANAEROBIC BACTERIA \*

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IN an earlier paper (1914) I published the results of a series of investigations into the importance of hydrogen-ion concentration in the hæmolytic process. These researches concerned a number of heterogeneous hæmolytic substances, namely opera lysin, vibrio lysin, staphylolysin, cobra lysin, bee lysin, saponin, sodium glycocholate and sodium oleate. For my experiments I used blood corpuscles from horses, oxen, sheep and rabbits. I was induced at that time to take up the question on a somewhat broader basis by the pioneer and fundamental work carried out by Sørensen (1909) on the importance of the hydrogen-ion concentration in the enzymatic processes, as well as by the later treatise of Michaelis and Skwirsky (1909-10) dealing with the importance of this factor in amboceptor-complement hæmolysis.

As was to be expected my investigations demonstrated that the hydrogen-ion concentration constituted—as it did for all other biological processes—a factor of exceptional importance, of an importance so great, indeed, that it was difficult to understand how it should ever have been possible to carry out hæmolytic measurements without making allowance for it. The explanation must be sought in the fact that the experimental mixtures used contained as a rule substances such as normal serum of adequate buffer content. On the other hand, it is hardly open to doubt that in a number of cases deficient buffer action was responsible for irregularities in the series of experiments ("experimental errors").

From these researches it emerged that the effect of the hydrogen-ion concentration is by no means identical for the different hæmolysins. Broadly speaking it may be of two types, type A, in which there is an optimal point with decreasing hæmolysis on both sides, and type B, with a point of minimal activity and increasing hæmolysis on both sides. In several cases, for example the combination

\* This paper is also published in the Sørensen jubilee volume of the *Comptes Rendus du Laboratoire Carlsberg* (1938, vol. xxii, Serie chimique).

staphylolysin-sheep blood, the types appear to be combined, as there exist points of both maximal and minimal activity.

The buffer substance employed for the bulk of my experiments was 2-3 per cent of the homologous serum. A batch of experiments carried out simultaneously showed, however, that the presence of other buffer substances (phosphates, peptone, egg albumen) is frequently capable of altering the shape of the curves in an even higher degree. Details of these conditions are set forth in the publications already mentioned.

The experiments on the importance of *pH* for the lysins of *B. uelchii*, *B. oedematis*, vibron septique and *B. tetani*, here under consideration, were made only with horse blood corpuscles to which normal horse serum or phosphate had been added as buffer

### Technique

The defibrinated blood was centrifuged and washed twice by centrifugation with 0.9 per cent NaCl solution. A series of mixtures (50 c.c. of each) was prepared containing washed blood corpuscles, a buffer in the form of serum or phosphate, and varying quantities of hydrochloric acid or sodium hydroxide as indicated below.

Buffer, normal serum		Buffer, phosphates	
Each mixture contains 25 c.c. of 4 per cent blood-corpuscle suspension + 0.5 c.c. normal horse serum + the indicated quantities of HCl or NaOH + 0.9 per cent NaCl to 50 c.c.		Each mixture contains 25 c.c. of 4 per cent blood-corpuscle suspension + 2.5 c.c. secondary phosphate (Sörensen) + 2.5 c.c. primary phosphate (Sörensen) + the indicated quantities of HCl or NaOH + 0.9 per cent NaCl to 50 c.c.	
Mixture no.		Mixture no.	
1	0.67 c.c. N/10 HCl	1	1.5 c.c. N/10 HCl
2	0.55 " " "	2	1.0 " " "
3	0.44 " " "	3	0.75 " " "
4	0.33 " " "	4	0.5 " " "
5	0.22 " " "	5	0.25 " " "
6	0.11 " " "	6	0
7	0	7	0.25 c.c. N/10 NaOH
8	0.11 c.c. N/10 NaOH	8	0.5 " " "
9	0.22 " " "	9	0.75 " " "
10	0.33 " " "	10	1.0 " " "
11	0.44 " " "	11	1.25 " " "
12	0.55 " " "	12	1.5 " " "
13	0.67 " " "	13	1.75 " " "
14	0.78 " " "	14	2.00 " " "
		15	2.5 " " "

To each of a series of calibrated test tubes (11 or 15) a quantity of blood corpuscles sufficiently large to cause partial hemolysis (about 20 per cent.) in a preliminary experiment under given conditions. This was added to 1 c.c. of 0.9 per cent NaCl solution and 9 c.c. of the buffer substance were subsequently added. The tubes were then placed in the water bath at 37° C. for 2 hours.

ogon shaken and kept in the ice cellar overnight. By means of the colorimetric method described by Madsen (1899), the quantity of hæmoglobin dissolved in each mixture was then ascertained.

Simultaneously with the series of experiments proper, a corresponding batch of control experiments without hæmolyisin was made in an identical manner. In determining the degree of hæmolytic action produced by the quantity of lysin at the various hydrogen ion concentrations I have deducted the "spontaneous" hæmolytic action in the control batch provided the hæmolytic action of the experiments proper was below 100 per cent. The admissibility of this manner of proceeding has been discussed at some length in my earlier paper.

The pH of the experimental mixtures was measured colorimetrically, the blood corpuscles in corresponding mixtures of lysin, blood corpuscles and acid or base being removed by centrifugation immediately after mixing and the measurement carried out on the clear and generally colourless solution.

The lysins were prepared by anaerobic incubation of the respective microbes in meat medium under liquid paraffin at 37° C during the periods indicated in the tables. As filtration through bacterio tight filters usually weakens the hæmolytic activity very considerably I confined myself to removing the greater part of the bacteria by centrifugation.

### Results

In cultures of *B. histolyticus*, *B. sporogenes*, *B. sordellii* and the bacillus of lamb dysentery it has not been possible to demonstrate the presence of lysins *vis-à-vis* horse blood corpuscles.

The details of the experiments are shown in tables I-IV and charts 1-5.

The experiments demonstrate that the role played by pH in the experimental mixtures is highly important for the action of these lysins. If we examine first the experiments for which homologous normal serum served as buffer substance, we find that the effect of pH is apparently of the same character for *B. welchii* and *B. adematensis* lysin and tetanolysin, as the hæmolytic action diminishes evenly with increasing pH, whereas the vibrio septique lysin behaves quite differently in this respect. We observe for this lysin a distinct point of minimum activity at about pH 6.8, with increasing hæmolytic action on both sides of it. Thus it corresponds to type B.

The substitution of phosphate buffer for normal serum does not alter the course of the process as far as *B. adematensis* lysin and tetanolysin are concerned, but it affects in no small measure that of the other two lysins, which both behave in the manner of type A, exhibiting optimal action at about pH 7.2-7.3 with decreasing hæmolytic action on both sides.

I do not propose to discuss here in detail the theoretical aspect of the course of these curves, as this is discussed in my earlier publication. I shall confine myself to stressing the fact that destruction of lysin caused directly or indirectly by certain hydrogen- and hydroxyl-ion concentrations may be of importance.



TABLE I (see chart 1).

B welchii (B perfringens) lysin, strain 9; 20 hours' culture.

Mixture	Serum blood mixture + 0.05 c c lysin				Phosphate-blood mixture + 0.005 c c lysin			
	pH	Hemolysis (per cent.)			pH	Hemolysis (per cent.)		
		Total	Control	Due to lysin		Total	Control	Due to lysin
1	6.35	48	2	46				
2	6.50	48	1	47				
3	6.60	42	1	41	6.50	31	15	16
4	6.70	37	1	36	6.64	28	8	20
5	6.85	33	1	32	6.80	29	5	24
6	7.00	32	1	31	6.95	30	4	26
7	7.10	28	1	27	7.03	30	3	27
8	7.20	26	1	25	7.20	29	2	27
9	7.35	22	1	21	7.30	27	2	25
10	7.50	18	1	17	7.40	27	2	25
11	7.65	15	1	14	7.55	25	2	23
12	7.75	11	1	10	7.65	20	2	18
13	7.85	9	1	8	7.85	18	2	16
14	8.00	8	2	6	8.05	15	3	12
15					8.40	6	6	0

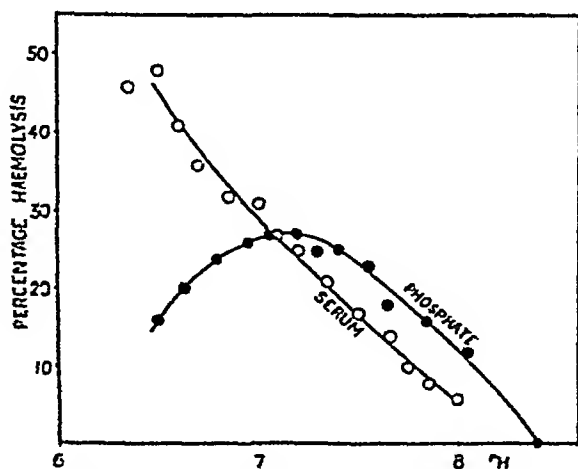
CHART 1.—The importance of the hydrogen-ion concentration for the action of *B. welchii* (*B. perfringens*) lysin.

TABLE II (see chart 2)

*Vibrio septique lysin*, strain 3, 18 hours' culture

Mixture	Serum blood mixture +0.15 c.c. lysin				Phosphate blood mixture +0.24 c.c. lysin			
	pH	Hæmolysis (per cent)			pH	Hæmolysis (per cent)		
		Total	Control	Due to lysin		Total	Control	Due to lysin
1	6.47	24	2	22			25	
2	6.60	22	1	21			10	
3	6.70	20	1	19			8	
4	6.80	20	1	19	6.75	0	5	4
5	6.90	20	1	19	6.85	11	3	8
6	7.00	24	1	23	6.95	13	2	11
7	7.10	25	1	24	7.05	15	2	13
8	7.20	28	1	27	7.15	17	2	15
9	7.30	30	1	29	7.25	19	2	17
10	7.40	33	1	32	7.35	20	2	18
11	7.50	33	1	32	7.45	18	2	16
12	7.60	30	1	29	7.60	14	2	12
13	7.70	40	2	38	7.70	12	2	10
14	7.80	45	2	43	7.85	10	3	7
15							60	

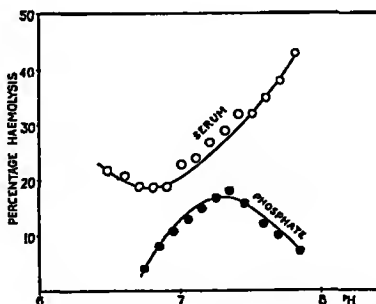
CHART 2—The importance of the hydrogen ion concentration for the action of *vibrio septique lysin*

TABLE III (see chart 3).

B. *œdematians* *lysin*, strain 7, 18 hours' culture.

Mixture	Serum blood mixture + 0.15 c c <i>lysin</i>				Phosphate-blood mixture + 0.1 c c <i>lysin</i>			
	pH	Hæmolysis (per cent)			pH	Hæmolysis (per cent)		
		Total	Control	Due to <i>lysin</i>		Total	Control	Due to <i>lysin</i>
1	6.30	60	2	58	6.25	70	4	66
2	6.40	52	2	50	6.40	55	3	52
3	6.50	50	2	48	6.50	52	2	50
4	6.65	41	2	39	6.60	48	1	47
5	6.80	38	2	36	6.70	35	1	34
6	6.90	35	2	33	6.80	29	1	28
7	7.00	32	2	30	6.95	22	1	21
8	7.10	30	2	28	7.05	18	1	17
9	7.20	27	2	25	7.20	12	1	11
10	7.35	24	2	22	7.25	12	1	11
11	7.40	23	2	21	7.40	7	1	6
12	7.50	19	2	17	7.50	5	1	4
13	7.65	16	2	14	7.65	6	2	4
14	7.80	14	2	12	7.85	3	2	1
15					8.00	2	2	0

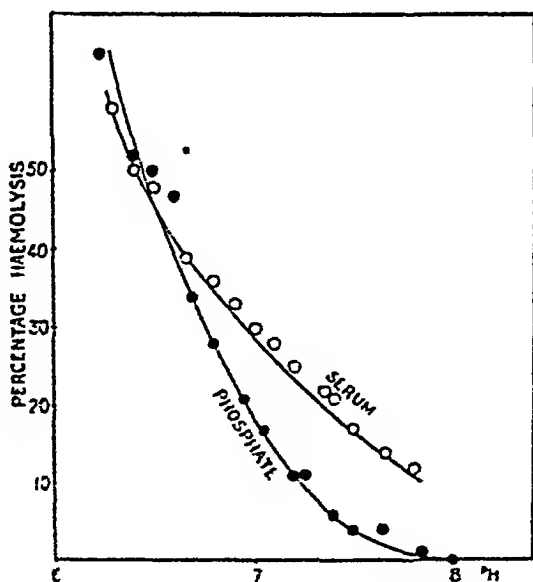
CHART 3—The importance of the hydrogen ion concentration for the action of *B. œdematians* *lysin*.

TABLE IV (see chart 1)  
*Tetanolysin*, 44 hours' culture

Mixture	Serum blood mixture + 0.01 c.c. lysin				Phosphate-blood mixture + 0.018 c.c. lysin			
	pH	Hemolysis (per cent.)			pH	Hemolysis (per cent.)		
		Total	Control	Due to lysin		Total	Control	Due to lysin
1	0.50	48	0	48			00	
2	0.75	48	0	48			35	
3	0.90	48	0	48	0.45	38	10	28
4	7.10	34	0	34	6.60	32	4	28
5	7.30	27	0	27	0.70	25	2	23
6	7.38	20	0	20	6.80	20	1	19
7	7.50	15	0	15	7.05	10	1	15
8	7.70	10	0	10	7.17	15	1	14
9	7.80	0	0	0	7.25	10	1	0
10	7.00	4	0	4	7.45	8	1	7
11	8.10	4	1	3	7.00	7	1	6
12	8.30	4	2	2	7.80	3	1	2
13			12		8.10	1	1	0
14			30		8.35	2	2	0
15							75	

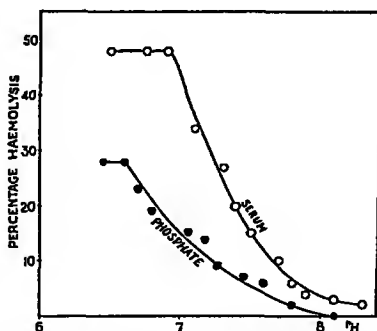


CHART 4—The importance of the hydrogen ion concentration for the action of *tetanolysin*

A number of experiments were also made with a view to investigating the effect of  $pH$  on the stability of the four lysins examined. These were carried out in the following manner. Varying amounts of hydrochloric acid or sodium hydroxide were added to lysin in a series of test-tubes and the  $pH$  measured colorimetrically. The mixtures were then placed in the water-bath for 2 hours at  $37^{\circ}C$ , neutralised with equivalent quantities of sodium hydroxide or hydrochloric acid and the volume adjusted uniformly with 0.9 per cent sodium chloride. By titrating in the usual manner (horse blood corpuscles with phosphate buffer  $pH$  6.8), the quantity of the different mixtures producing the same partial hæmolysis (25 per cent) was determined. The results of these experiments are exhibited graphically in chart 5.

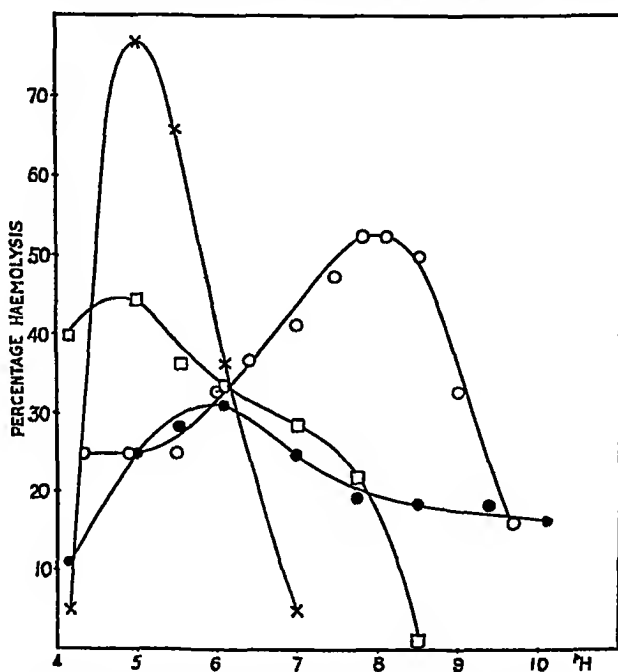


CHART 5—Stability of the hæmolysins at varying  $pH$ .

- |   |                                      |
|---|--------------------------------------|
| □ - - - <i>B. welchii</i> ( <i>B. perfringens</i> ) lysin | × - - - <i>Vibrio septique</i> lysin |
| ● - - - <i>B. œdematens</i> lysin                         | ○ - - - Tetanolysin                  |

The points of optimum stability thus appear to be

for <i>B. welchii</i> ( <i>B. perfringens</i> ) lysin	about $pH$ 5.0
„ <i>vibrio septique</i> lysin	„ „ 5.0
„ <i>B. œdematens</i> lysin	„ „ 6.0
„ tetanolysin	„ „ 8.0

A glance at charts 1-4 will show that a lysin destruction, of this kind at any rate, can hardly be responsible for the course of

the tetanolysin curve (chart 1) or for that of either the *welchii*-phosphatase curve or either of the vibron septique curves, whereas such a possibility exists as regards the *welchii*-normal serum curve and both the curves for the *B. adematensis* lysin

### Summary

1 In this work the effect of pH on the hæmolysis of horse blood corpuscles by the lysins of *B. welchii*, vibron septique, *B. adematensis*, and *B. tetani* is examined. As the hydrogen-ion concentration plays a very important rôle in the course taken by the hæmolytic process, it must be considered essential that, when quantitative researches on hæmolysin are carried out, this factor should be kept constant in the experimental mixtures by the addition of suitable buffer substances.

2 The point of maximum stability for the various toxins has been determined.

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# THE TOXICITY OF SOME METHYL DERIVATIVES OF BENZENE WITH SPECIAL REFERENCE TO PSEUDOCUMENE AND HEAVY COAL TAR NAPHTHA \*

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It was shown by Cameron *et al* (1937) that certain chlorine derivatives of benzene, especially o-dichlorobenzene, which had proved valuable in the campaign against the bed bug (*Cimex lectularius* Linn) were toxic to laboratory animals. The opinion was expressed that it was inadvisable to expose human beings to o-dichlorobenzene. In searching for more suitable agents against the bed bug, after conferring with Messrs Ashmore and McKenny Hughes it was decided to investigate some of the methyl derivatives of benzene. Tattersfield and Roberts (1920) had already shown the efficacy of pseudocumene (1,2,4-trimethylbenzene) against wireworms. Preliminary experiments by Thomas and McKenny Hughes with bed bugs gave promising results and led to the extensive investigation of Ashmore and McKenny Hughes (1937). These workers obtained excellent results in the field, both with technical pseudocumene and a heavy coal tar naphtha containing about 30 per cent pseudocumene. Moreover they showed that with effective ventilation heavy naphtha rapidly disappears from rooms even after the maintenance for 24 hours of an air concentration of 0.12–0.15 per cent. Eight hours' ventilation was sufficient to reduce the concentration to 0.009 per cent V/V and there was no further "building up."

The importance of knowing whether these compounds were injurious to experimental animals and presumably in that case to man was recognised from the start. Laboratory experiments were therefore carried out both on pseudocumene and heavy coal tar naphtha and on some of the simpler methylated benzenes. This paper reports the results of these experiments.

\* Work carried out for the Bed Bug Infestation Committee of the Medical Research Council.

† Graham Scholar.



### Methods

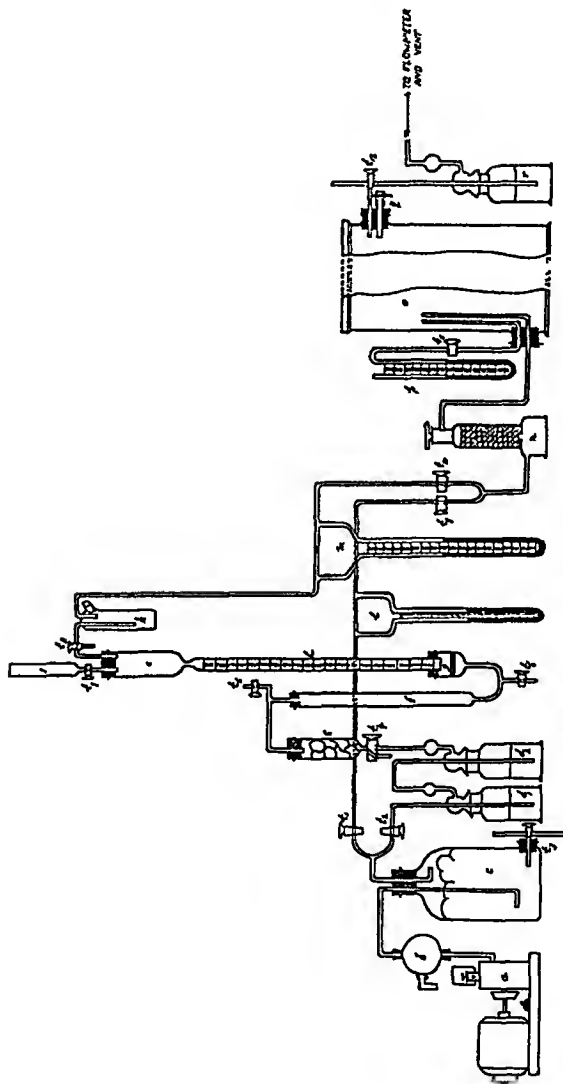
We have worked with fully grown male and female albino rats, guinea-pigs, mice and rabbits. Most of the rats were of the Wistar strain. In all about 700 animals, chiefly rats and mice, were used. The effects produced by the subcutaneous and intraperitoneal injection, oral administration, inhalation and painting on the skin of toluole of *o*-, *m*- and *p*-xylole, mesitylene, pseudocumene and heavy coal tar naphtha have been studied. At the conclusion of the experiment the animals were killed with ether or a sharp blow on the head and pieces of lung, liver, kidney, spleen, adrenal and occasionally brain and subcutaneous tissue from the site of injection were fixed at once in 10 per cent formol-saline and 70 per cent alcohol. Paraffin sections were stained with Ehrlich's acid hæmatoxylin and eosin and by van Gieson's method, frozen sections with Scharlach R. A complete blood examination was carried out by one of us (J L H P) on a series of rabbits before and after subcutaneous administration of the substances under investigation. This included both total red and white cell counts using a Burkner counting chamber, hæmoglobin estimation using the Haldane method, platelet and reticulocyte counts with supravital brilliant cresyl blue staining and a differential leucocyte count on smears stained by Jenner's method.

For inhalation experiments we used practically the same apparatus as described by Cameron *et al* for chlorinated benzenes. In view of the higher volatility of pseudocumene, compared for example with that of *o*-dichlorobenzene, the saturation unit was redesigned to ensure complete saturation. The principle of the new saturator is shown in the accompanying figure. The air is first bubbled through two wash-bottles ( $d_1$  and  $d_2$ ) containing the liquid and then through a tube packed with cotton wool ( $e$ ) which has been well soaked in the liquid. This tube however may be omitted. The air is then split up by being passed through a sintered porous glass plate ( $g$ ) and is bubbled upwards through a 1 metre column of the liquid ( $h$ ). In a number of experiments checked by chemical analysis of the air in the gas chamber, the final figure for the saturated air with the new saturator was within 1 part in 5000 of the theoretical figure. The apparatus was accurate down to 50 parts per million and maintained that accuracy during a continuous run of 48 hours.

It is our opinion that the comprehensive laboratory investigation of a supposedly toxic agent should attempt to determine the following:

- 1 The smallest fatal and lowest toxic dose for a variety of animals by various routes of administration
- 2 The nature of the pathological changes in tissues and blood produced by toxic doses.
- 3 Differentiation between acute and chronic effects
- 4 Effect on the growth of animals of prolonged exposure to the toxic agent.
- 5 Influence on reproduction, especially interruption of pregnancy and foetal changes
- 6 Acquisition of tolerance (resistance) or susceptibility
- 7 The factors which may modify the toxic action, *e.g.* fatigue, general nutrition—especially degree of fatness, sex and age
- 8 Preventive, protective or curative measures which may be directed against any toxic effects.
- 9 Interpretation of results in terms of hazards occurring in industry, etc.

So far as the methylated benzenes are concerned, a great deal of information already exists in relation to items 8 and 9 and is fully discussed by Alice Hamilton (1925), Legge (1931) and Ethel Browning (1937). This paper deals with some of the remaining points.



Apparatus for exposing small animals to methylated benzenes

- |                                 |   |   |  |
|---------------------------------|---|---|--|
| a                               | Electric power blower   | g | Animal chamber   |
| b                               | Pressure valve  | h | 1 Lr. urethane bottle  |
| c                               | Aspirator bottle packed with cotton wool for filtering air from motor | i | Outlet to wash bottle with sampling tube                         |
| d <sub>1</sub> , d <sub>2</sub> | Wash bottles containing the liquid under vacuum                       | j | Wash bottle containing alcohol for absorption of liquid from air |
| e                               | Tubes with cotton wool soaked in the liquid                           | k | Stopcocks for collecting samples of gas mixture for analysis     |
| f                               | Mixing tower filled with glass beads                                  |   |  |
| g                               | Sintered porous glass plate   |   |  |
| h                               | Metre column of the liquid  |   |  |
| i                               | Wash bottle for trapping bubbles from h                               |   |  |
| j                               | Trist for filling h   |   |  |
| k                               | Wash bottle for collecting droplets of liquid carried over from h     |   |  |
| l and m                         | Y-tube meters   |   |  |
| n                               | Mixing tower filled with glass beads                                  |   |  |

## RESULTS

## Heavy coal tar naphtha

The heavy coal tar naphtha samples used in these experiments were obtained from a number of firms and satisfy the specifications drafted by Ashmore and McKenny Hughes, viz

- " 1 *Colour*—Shall not be darker than a freshly prepared solution of 1 ml of N/10 iodine in 1000 ml of distilled water
- 2 *Specific Gravity*—Not less than 0.835 and not greater than 0.910.
3. *Water*—Shall be free from water and other visible impurities at 15.5° C
4. *Distillation Range*.—Method as defined in B S I. Specification No. 479 for Coal-tar Naphthas  
Up to 160° C not more than 5 ml  
Up to 190° C not less than 90 ml
- 5 *Flash-point (Abel)*—Not less than 105° F
6. *Tar Acids and Tar Bases*—Not more than 0.25 per cent of tar acids and 0.25 per cent of tar bases "

The main constituents of heavy coal tar naphtha are the trimethyl benzenes—pseudocumene (1, 2, 4-trimethylbenzene, B P. 169.8° C), hemimellitol (1, 2, 3-trimethylbenzene, B P. 175° C) and mesitylene (1, 3, 5-trimethylbenzene, B P. 164.5° C). There are present in addition propyl benzene (B P. 159° C), tetramethylbenzene (B P. 192° C), cumarone (B P. 169° C), indene (B P. 178° C) and methyl ethyl benzene (B P. 159° C), with small amounts of naphthalene (B P. 218° C), tar acids (phenolic) and tar bases (dimethylpyridenes). Possibly also there are minute traces of trimethylthiophene. Pseudocumene and mesitylene constitute about 65 per cent of heavy coal tar naphtha.

The animal experiments are summarised in tables I-IV

TABLE II

*Subcutaneous administration of heavy naphtha \* to rats*

Number of animals	Amount injected	Killed after	Results	
3	1.0 c.c.	24 hours	Organs healthy	necrosis at
3	0.5 "	"	injection site	necrosis at
3	0.1 "	"	Organs healthy	necrosis at
3	0.05 "	"	injection site	necrosis at
3	0.01 "	7 days	Organs healthy	injection site
3	0.005 "	"	Organs and tissues healthy	"
3	0.001 "	"	" " "	" "
			" " "	" "

Body-weight of rats varied from 120 to 220 g. The maximum dose corresponded to 5 g per kg body-weight.

\* Gas Light and Coke Co. naphtha

*Conclusion.* Rats are resistant to large amounts of heavy naphtha administered subcutaneously.

TABLE I—Toxicity tests on 8 samples of heavy coal tar naphtha (temp 20° C)

Source	Concentration (per cent)	Exposure time	Necrosis (per cent.)	Mortality		Morbidity	
				No	Per cent	Liver	Kidneys
Rats							
Gas Light and Coke Co (1)	0.18	3 months	0	0	0	0	0
" " Co Bristol (2)	0.18	50 hrs	0	0	0	0	0
Dorman, Long & Co (highly refined)	0.18	21 "	0	0	0	0	0
" " " (semi refined)	0.18	27.5 "	0	0	0	0	0
Yorkshire Tar Distillers Ltd	0.18	21 "	0	0	0	1 slight fatty	0
Scottish Tar Distillers Ltd	0.18	31 "	0	0	0	2 slight fatty	0
Carlisle, Capel and Leonard, London *	0.18	27 "	0	0	0	0	2 slight fatty
	0.16 0.18	25 "	0	0	0	1 fatty	1 fatty
Mice							
Gas Light and Coke Co (1)	0.18	28 1/2 hrs	0	0	0	0	0
" " Co Bristol (2)	0.18	50 "	0	0	0	0	0
Dorman, Long & Co (highly refined)	0.18	21 "	0	0	0	0	0
" " " (semi refined)	0.18	27 1/2 "	0	0	0	0	0
Yorkshire Tar Distillers Ltd	0.18	21 "	0	0	0	0	0
" " " "	0.18	34 "	37	1	27	2 fatty	1 fatty
Scottish Tar Distillers Ltd	0.05	48 "	0	0	0	Necrosis in 1, others fatty	Marked fatty change in 7
Carlisle, Capel and Leonard, London *	0.18	27 "	13	0	0	3 fatty	3 fatty
" " " "	0.16 0.18	25 "	20	2	13	2 slight fatty	2 slight fatty
" " " "	0.05	48 "	0	0	0	0	0

\* Kindly supplied by Dr H Taylor of Imperial Chemical Industries Ltd

5 rats exposed to Gas Light and Coko Co (1) naphtha, 15 minutes in each of remaining groups

**Conclusion** Rats are highly resistant to heavy coal tar naphtha vapour and mice are susceptible only to high concentrations of some samples. Such lethal and toxic concentrations are far higher than any met with in reoccurring houses after disinfection.

TABLE III

*Inhalation of air saturated with heavy naphtha \* (temp 15-20° C)*

Animals	Number used	Period of exposure	No which survived	Killed after	Results
Rats	6	12 hours	6	12 hours	Organs healthy
"	12	24 "	12	1-3 days	" "
Mice	12	28½ "	12	24 hours	" "
Guinea-pigs	6	21½ "	6	24 "	" "
Rabbits	3	12 "	3	3 days	" "

The body-weight of the rats varied from 150-200 g, mice averaged 20 g, guinea-pigs 200-300 g, rabbits 2 kg Both males and females were used The animals fasted during the experiments

\* Gas Light and Coke Co naphtha

*Conclusion* Rats, mice, guinea-pigs and rabbits exposed for 12-28 hours to air saturated with heavy naphtha experience no ill effects and show no evidence of damage to their organs or tissues.

TABLE IV.

*Prolonged exposure of rats to air saturated with heavy naphtha \* (temp 15-20° C)*

Rat no	Period of exposure	Weight		Behaviour and appearance	Results
		at commencement	at termination		
1	2 months	142 g	215 g	Active, healthy	Organs healthy
2 †	"	130 "	230 "	" "	" "
3 †	"	146 "	250 "	" "	" "
4	"	144 "	250 "	" "	" "
5	"	154 "	220 "	" "	" "

\* Gas Light and Coke Co naphtha

† Female, gave birth to six healthy young rats one month after exposure began

‡ Female, pregnant at cessation of experiment, obviously this animal had become pregnant during the experiment

*Conclusion* Rats can be exposed for two months to air saturated with heavy naphtha without showing any diminution in their rate of growth or activity There is no evidence of damage to their organs or tissues after such exposure nor of interference with reproduction.

In addition, rats were given 0.12-0.25 c.c. heavy naphtha by mouth All survived, although those receiving the larger doses showed evidence of shock and considerable respiratory embarrassment for half to one hour afterwards Their organs on examination 24 hours later were healthy. Painting extensive skin surfaces with heavy naphtha produced in rats and rabbits evidence of

considerable local irritation, which however was temporary. There was no evidence of damage to the organs 24 hours after such treatment.

With rabbits 2 kg in weight, intravenous administration of 0.1–0.4 cc of heavy naphtha produced neither immediate nor delayed disturbance. The organs and tissues appeared quite healthy after such doses.

*General conclusions* In these experiments the animals were subjected to a very severe test, namely either large doses administered subcutaneously or exposure to air saturated with heavy naphtha at room temperature (15–20° C). Such conditions are far in excess of anything likely to be encountered in the field. It appears then that small laboratory animals are highly resistant to heavy naphtha whether administered in huge doses subcutaneously or by exposure for short or prolonged periods to air saturated with naphtha.

#### Pseudocumene and mesitylene

Pseudocumene (1,2,4-trimethylbenzene) and mesitylene (1,3,5-trimethylbenzene) are present in coal tar naphtha. Both are colourless liquids whose boiling points are 169.8° and 164° C respectively. Separation on a large scale by fractional distillation is therefore extremely difficult.

The technical pseudocumene used in these experiments was obtained from British Drug Houses, Ltd. Mr S. A. Ashmore has supplied us with the following information about it:

Specific gravity at 60° F	0.865
Refractive index at 20° C	1.489
Boiling range	163°–169° C
Flash point (Abel closed)	111° F
Concentration of vapour in air saturated at 60° F	0.19 per cent volume/volume

The sample we worked with contained approximately 70 per cent of crude aromatics of the pseudocumene-mesitylene type. A pure sample of mesitylene (BP 164° C) was supplied by British Drug Houses Ltd.

Rats tolerate large doses of pseudocumene and mesitylene subcutaneously, as much as 2 cc (12 cc/kg body weight) having been administered without fatal results. On the other hand, 1.5–2.0 cc intraperitoneally resulted invariably in death within 24 hours.

Pseudocumene when inhaled produced no ill effects. Even after 48 hours' continuous exposure to air saturated with pseudocumene and after 14 exposures of 8 hours each, rats appeared unaffected and microscopical examination of their organs failed to disclose any pathological change.

Mesitylene, however, proved toxic in high concentrations (table V). Thus of 16 rats exposed to air saturated with mesitylene (12 mg per litre or 2240 p.p.m.) for 24 hours 4 died. Their organs showed no change, apart from congestion of the lungs. The animals slowly became narcotised and died from respiratory failure. Twenty-four hours' continuous exposure to 1/4 saturation (560 p.p.m.) produced no ill effects, neither were rats affected after 14 days' exposure to a concentration of 560 p.p.m. for 8 hours daily. Mice were unaffected by 14 days' exposure to 560 p.p.m. of mesitylene.

Prolonged exposure to the lower concentrations of pseudocumene and mesitylene did not interfere with growth. Several female rats gave birth to healthy offspring whilst exposed to pseudocumene. These young rats grew normally when separated from their mother.

*Conclusion.* We believe therefore that pseudocumene is innocuous to laboratory animals, whilst mesitylene in high concentrations may be toxic.

#### Toluole and the xyloles.

Since a fair amount of work has already been done on toluole and the xyloles we need only summarise our results, which are in fair agreement with those of previous workers (Browning).

With subcutaneous injection the lethal dose for toluole was 5-10 c.c. per kg., for p- and m-xylole 5-10 c.c. per kg. and for o-xylole 2.5-5 c.c. per kg. With intraperitoneal injection the lethal dose for toluole was 2.0 c.c. per kg., for p- and m-xylole 2.2-5 c.c. per kg. and for o-xylole 1.5-2 c.c. per kg. body-weight. With inhalation (table V), exposure to 12,000-24,000 p.p.m. of toluole proved fatal to rats and mice in 1½-6 hours, whereas 24 hours' exposure to 6100 p.p.m. or 14 exposures of 8 hours each to 1525 p.p.m. was without effect.

In the case of the xyloles, o- and m-xylole appeared equally toxic, concentrations of 2000-3000 p.p.m. killing animals after 24 hours' exposure. Mice appeared to be more sensitive to m-xylole than rats. p-Xylole, on the other hand, was less toxic than the others. The organs of animals dying after exposure to toluole or xylole showed no characteristic changes.

Very young rats were more resistant to toluole than adult animals. Thus 33 per cent. of a group of 12 rats 9 days old survived 5½ hours' exposure to air saturated with toluole in contrast to 100 per cent. mortality within 5½ hours in a group of adult rats.

*Conclusion.* On the whole the xyloles appear to be somewhat more toxic than toluole, but there is not a great deal of difference between any of them. However with concentrations approximating

TABLE V. *LD<sub>50</sub> of m-Xylene (100%) in Mice (g), 20°C*

Toluene							
Saturation	100	244.5	10 rats	12	1	0	
"	100	244.5	10 mice	12	1	0	
"	100	244.5	10 rats	6	10	100	
"	100	244.5	10 mice	6	10	100	
"	50	122.25	10 rats	6	1	0	
"	50	122.25	10 mice	6	10	100	
"	25	61.125	10 rats	24	0	0	
"	25	61.125	10 mice	24	0	0	
"	12.5	30.5625	10 rats	8-14	0	0	
o-Xylene							
Saturation	5	12.5	10 rats	12	2	20	1 died after 24 hrs
"	5	12.5	10 mice	12	2	20	1 " " "
"	2.5	6.25	10 rats	24	0	0	
"	2.5	6.25	10 mice	24	0	0	
"	1.25	3.125	10 rats	24	1	10	8 died in 22 hrs
"	1.25	3.125	10 mice	24	4	40	
"	0.625	1.5625	10 rats	24	0	0	
"	0.625	1.5625	10 mice	24	0	0	
"	0.3125	0.78125	10 rats	8-14	0	0	
m-Xylene							
Saturation	25	5010	10 rats	12	1	10	
"	25	5010	10 mice	12	0	0	4 dead in 7 hrs
"	25	5010	10 rats	24	0	0	
"	25	5010	10 mice	24	0	0	Died 4 days after exposure
"	12.5	2505	10 rats	24	0	0	
"	12.5	2505	10 mice	24	0	0	
"	12.5	2505	10 rats	8-14	0	0	
p-Xylene							
Saturation	10	19650	10 rats	12	8	60	1 dead after 6 hrs
"	10	19650	10 mice	12	0	0	6 " " "
"	5	9825	10 rats	24-28	0	0	
"	5	9825	10 mice	24-28	0	0	
"	2.5	4912.5	10 rats	24	0	0	
"	2.5	4912.5	10 mice	24	0	0	
"	1.25	2456.25	10 rats	24	0	0	
"	1.25	2456.25	10 mice	24	0	0	
"	0.625	1228.125	10 rats	8-14	0	0	
"	0.625	1228.125	10 mice	8-14	0	0	
Mesitylene							
Saturation	12	2240	10 rats	24	4	25	
"	6	1120	10 mice	24	0	0	
"	6	1120	10 rats	24	0	0	
"	6	1120	10 mice	8-14	0	0	
"	3	560	10 rats	8-14	0	0	
Pseudocumene							
Saturation	10.8	1800 2000	8 rats	12	0	0	
"	10.8	1800 2000	10 mice	12	0	0	
"	10.8	1800 2000	4 rats	48	0	0	
"	10.8	1800 2000	0 "	8-14	0	0	
"	2.7	450 500	0 "	24	0	0	



those possible under industrial conditions even prolonged exposure failed to produce any serious effects.

### *Blood changes*

The results of the blood examination of representative rabbits are shown in table VI. No definite effect has been produced, the variations noted being well within the limits of normality for rabbits laid down by Pearce and Casey (1930). Although a number of observers (Selling, 1910, Batchelor, 1927; Woronow, 1929, Mgebrow, 1930; Farber, 1933; Engelhardt, 1935) have described alterations in the blood after exposure to toluole, xyloles and pseudocumene, a careful scrutiny of their figures when given inspires little conviction, since no attention has been paid to normal variations. Neither has any worker considered the possibility of spontaneously developing blood diseases. Professor A. E. Boycott tells us that he has several times seen a severe anæmia develop in a rabbit apparently spontaneously. One such instance, largely a dilution anæmia, was studied by Boycott and Douglas (1908-09). In two of our rabbits severe anæmia of the aplastic type developed before the experiment commenced. If these had not been recognised the condition might well have been attributed to one of the agents studied.

### CONCLUSIONS.

The order of toxicity of the substances under investigation appears to be

heavy naphtha and pseudocumene < mesitylene < toluole < xyloles.

Our results may be summarised as follows:—

#### **Toluole.**

12,200 p p m —fatal to rats and mice in 6½ hours

1,525 „ —rats survived 14 exposures of 8 hours each

#### **o-Xylole**

6,125 p p m —fatal to rats and mice in 12 hours

3,062 „ —fatal to rats and mice in 24 hours

1,530 „ —rats survived 14 exposures of 8 hours each

#### **m-Xylole.**

8,040 p p m —fatal to rats and mice in 24 hours

2,010 „ —fatal to mice in 24 hours, rats survived

1,005 „ —rats survived 14 exposures of 8 hours each

#### **p-Xylole.**

19,650 p p m —fatal to rats and mice

4,912 „ —rats and mice survived 24 hours' exposure

1,226 „ —rats and mice survived 14 exposures of 8 hours each

TABLE VI

Blood changes following the subcutaneous injection on 3 consecutive days of 1 cc of some methylated benzenes (representative rabbits)

	Hb	R B C (millions per c mm )	W.B.C	Platelets	GI	Reticu- locytes (per cent )	Differential count				
							P	L	M	E	B
Toluene											
7 days before	71	4.5	0,000	454,000	0.81	0.8	45	51	1	1	2
4 " "	74	5.1	4,400	375,000	0.72	1.6	31	63	3	0	3
2 " after	72	5.48	8,100		0.66	0.0	27	63	2	2	0
4 " "	72	4.05	2,000		0.73	0.0	36	60	2	0	2
9 " "	74	4.51	8,900		0.80	0.0	15	76	3	0	6
o-Xylene											
0 days before	82	5.8	6,400	185,000	0.70	0.0	31	63	2	0	4
1 day "	84	5.4	0,400	518,000	0.77	0.8	27	70	0	0	3
2 days after	70	5.4	5,100	639,000	0.64	1.0	25	73	0	0	2
4 " "	62	4.4	6,000	501,000	0.70	1.8	50	50	0	0	0
0 " "	50	4.6	4,700	1,500,000	0.60	2.2	52	35	10	0	3
13 " "	57	4.1	5,300	490,000	0.69	3.0	49	43	2	2	4
m-Xylene											
8 days before	80	6.8	0,260	353,000	0.59	0.2	18	82	0	0	0
6 " "	78	6.0	8,000	400,000		0.8	24	70	2	0	4
1 day after	72	5.5	4,000	480,000	0.65	1.8	32	58	4	1	5
3 days "	70	5.2	7,000	582,000	0.67	1.0	31	50	7	1	2
12 " "	74	5.6	11,300		0.06	0.0	25	66	4	0	5
21 " "	71	6.52	8,100		0.64	0.0	30	62	4	2	2
p-Xylene											
11 days before	72	5.2	4,200	400,000	0.60	1.4	19	74	2	1	4
5 " "	78	5.4	3,500	475,000	0.72	3.2	26	57	3	0	14
2 " after	71	5.2	10,100	475,000	0.69	2.6	15	76	6	0	3
5 " "	7	5.7	14,000	775,000	?	1.2	24	66	1	1	8
8 " "	62	5.4	9,300	777,000	0.58	3.0	30	53	2	0	6
13 " "	70	5.0	6,100	380,000	0.62	0.6	12	81	2	0	5
28 " "	74	4.91	9,300		0.75	0.0	40	57	2	0	1
Mesitylene											
26 days before	74	5.04	7,300	220,000	0.74	0.0	32	60	4	2	2
10 " "	80	6.0	4,200	300,000	0.60	0.6	56	37	4	2	1
2 " after	82	5.4	4,000		0.70	0.0	27	71	1	0	1
4 " "	74	5.0	6,000		0.74	0.0	11	77	7	1	4
0 " "	76	5.28	3,300		0.72	0.0	28	60	4	0	2
16 " "	75	5.8	2,800		0.63	0.0	13	70	5	0	12
Pseudocumene											
20 days before	80	5.24	4,700	353,000	0.70	1.0	28	67	1	2	2
24 " "	74	5.2	4,700	350,000	0.71	0.2	60	34	2	1	3
2 " after	71	5.8	7,300		0.61	0.0	37	58	2	3	0
5 " "	68	5.2	13,300		0.65	0.0	28	64	6	0	2
0 " "	70	5.28	6,600		0.07	0.0	34	59	2	2	3

**Mesitylene.**

2,240 p p m —fatal to rats in 24 hours

560 „ —rats and mice survived 14 exposures of 8 hours each.

**Pseudocumene.**

1,800–2,000 p p m.—rats survived 48 hours' continuous exposure or 14 exposures of 8 hours each

**Heavy coal tar naphtha**

3,200 p.p m —rats survived continuous exposure for 2 months  
Some samples were toxic to mice.

Pseudocumene and most samples of heavy naphtha are without toxic effect on laboratory animals, mesitylene is toxic in high concentrations, probably only when the air is saturated with it, whilst toluole and the xyloles have a fairly wide range of toxicity

Prolonged exposure to concentrations comparable with those occurring in industry (1000 p p m for toluole and xyloles according to Batchelor, probably much lower for the other members of the group) leads to no ill effects. Growth proceeds normally and the offspring of exposed females are healthy. No characteristic changes are found in the organs of animals poisoned by the toxic compounds. Death occurs with increasing muscular weakness, narcosis and respiratory failure. There is no evidence of a specific action on the hæmatopoietic system

We know of no cogent reason why heavy coal tar naphtha or pseudocumene as specified in this paper should not be used under conditions involving human exposure

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the nature of this tissue and from their drawing no diagnosis is possible. Holton (1929-30), implanting liver primordia or pieces of liver from embryos of 3-6 days' incubation, found that liver developed up to the 12th day of incubation as a non-lobulated mass of normally developed tubules and sinusoids. At this time degenerative changes occurred, accompanied by an infiltration with granulocytes, and by the fourteenth day no liver was left. No gall-bladder was distinguished. Holton considered that the sudden change in the character of the transplant at twelve days was due to a change in the metabolic methods of the liver cells at that date.

Willier and Rawles (1930, 1931 *a* and *b*), and Willier, Rawles and Rudnick (1931), grafting early blastoderms, found that the liver consisted of an interlacing mass of liver cell cords and sinusoids without lobes or lobules, connected in some grafts with the gut by an epithelial tube which they considered to be the gall bladder. Liver did not as a rule develop in the absence of heart. This was confirmed by Rudnick (1932). R. H. Sandstrom \* (1934) (*née* R. G. Holton), in grafts of gut and liver from embryos of 3-6 days' incubation, found that liver up to 90 hours old at the time of grafting grew as a mass of liver cell cords connected with a structure representing the gall bladder and corresponding very well with embryonic liver at the same stage of development. Liver grafted when 6 days old showed enlargement, but was cystic and degenerate. By the eleventh day all grafts were densely infiltrated with granulocytes. She quotes Willier as having stated in a personal communication that "in chorio-allantoic grafts of liver tissue from hatched chick donors, only the bile ducts remain in the midst of an intense infiltration of leucocytes, the liver cells apparently having undergone disintegration" (R. H. Sandstrom, p. 239). She concludes that, as degeneration is less marked when gut is included in the graft, it is due to inability to function, and compares it with the corresponding degeneration of glandular tissue in the pancreas after ligation of the duct, where the islets, whose function is not interfered with, survive.

### Methods

Since autoplasmic transplants were clearly out of the question, homoio-plasmic transplants were carried out, using chicks of 18 days' incubation. The chick is removed from the egg in a sterile manner, the liver excised and cut into fragments from 2 to 4 mm across†. As hosts, eggs of 10 days' incubation were used. After the production of an artificial air sac as recommended by Burnet (1933), pieces of liver are blown on to the chorio-allantois with sterile Pasteur pipettes. The hole in the shell is then covered with a sterile coverslip and luted with paraffin wax. The eggs are returned to the incubator and the transplants removed for histological examination at intervals varying from one hour to ten days.

After a few trials it was found that Regaud's fixative used as recommended by Duthie (1935) gave the best results. Paraffin sections were stained with Ehrlich's haematoxylin and eosin and with Weigert's iron haematoxylin and van Gieson's stain for general topographical purposes. Frozen sections were stained with Sudan IV for fat and by McIndoe's method for bile capillaries. A few transplants were fixed in 96 per cent alcohol and paraffin

\* R. H. Sandstrom's extracts from the literature, when not positively inaccurate, are extremely misleading and should always be verified.

† There seems to be a prejudice in favour of small transplants. If however in the early stages a constant depth of cells survives, the larger the transplant within reasonable limits the better. Moreover if vascularisation is excited by the products of autolysis, a large transplant stands a better chance of vascularisation than a small one. Last but not least, large transplants are easier to find.

not only stained by Best's method for glycogen. For additional details of methods recommended by Duthie printed in *ibid.* 1936, p. 50.

# 1. HOMOLOGASTIC GRAFTS

## 17. *and* *18-day embryo chick liver grafts*

The structure of the grafted material is shown in fig. 1. The liver lobules are poorly separated from one another, their limit being indicated only by the portal canals. The liver cells are distended with fat, the bile ducts few, small and inconspicuous.

Up to about 4 hours the transplants appear well preserved though as early as one hour the mitochondria show variation in size and distribution greater than that seen in the original grafted material. By 4 hours cell marked pyknosis and fragmentation of the nuclei can be made out and one transplant at this time already showed mitochondrial separation into three zones: (1) an outer





among the surviving liver cells are trabecular masses of eosinophil liver cells showing such an increase of mitochondrial substance that no separate mitochondria can be made out. No bile ducts have been observed.

At 2 days (fig. 4) the transplant has sunk down into the chorio-allantois, which closes round it to form a bag (*cf* Wilher, 1924, "incorporation of the graft"). Most grafts show otherwise little advance on the previous day, others show irregular nodules of liver cells replacing the continuous ring. Slight signs of activity are occurring on the part of the membrane, including cedema and increased vascularity and cellularity.

By the third day (figs. 5, 6 and 7) the liver cells are arranged in small groups near the surface of the transplant and show slight cyst formation. In that part of the transplant which was originally apposed to the membrane are a few isolated cysts whose nature is rather doubtful. I am inclined to regard most of them as composed of liver cells because of their irregularly shaped lumen, their rounded nuclei, their numerous vacuoles (no vacuoles have ever been observed in the unquestioned bile ducts which are identifiable in older transplants) and the apparent transition between them and undoubted liver cells. Against this and in favour of their being bile ducts is the elongation of many of the nuclei, and the extremely small number of mitochondria. In the larger cysts, owing to the flattening of the epithelium, no distinction is possible.

Calcification first appears at this time. It occurs in all transplants of greater age than two days. Occasionally the whole transplant dies, but usually nodules of liver survive. In either case multiple tiny granules of calcium salts fill the degenerating liver cells, usually preserving their outlines but never extending far into the transplant. Later the calcified patches coalesce to form larger masses which may fill much of the centre of the transplant (figs. 12 and 13). Reaction on the part of the membrane is now well defined, though never very marked. Edema and cellular infiltration occur and some invasion of the graft by capillaries and fibroblasts. There is usually some hæmorrhage, often with "pseudopodial" tracking along the membrane, and green staining round the transplant. Proliferation and vacuolation of the cells of the membrane occur often, with keratinisation and cell nest formation. No leucocytic ring (Cameron and Oakley) is ever seen.

On the fourth day (figs. 8 and 9) the trabeculation of the liver cells is very marked and growth is evident, though no mitoses have ever been seen. Bile ducts are few and far between, usually far away from liver cells and easily distinguished from them. Bile capillaries can occasionally be demonstrated in the liver trabeculae and small cysts are common. In one transplant a large mass of erythropoietic tissue was present (fig. 5).

## CHORIO ALLANTOIC LIVER GRAFTS



FIG 1—Normal 18 day embryonic  
chick liver  $\times 66$



FIG 4—18 day chick embryonic liver  
2 days  $\times 66$

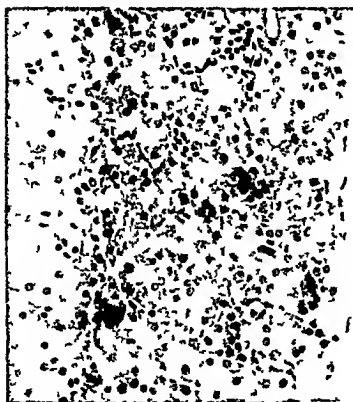


FIG 3—Histological detail of edge of  
fig 2  $\times 210$



FIG 5—18 day chick embryonic liver  
3 days Note mass of erythropoietic  
tissue  $\times 24$

All sections stained with Ehrlich's haematoxylin and eosin



## CHORIO ALLANTOIC LIVER GRAFTS

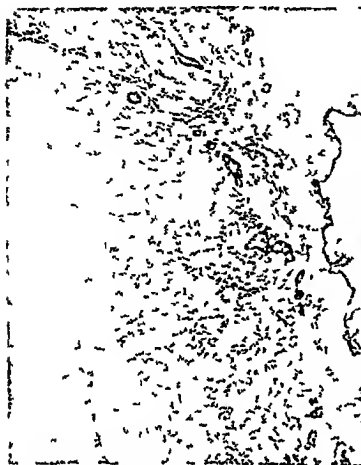


FIG. 6—18 day chick embryonic liver  
3 days  $\times 66$



FIG. 7—18 day chick embryonic liver 3 days  
Detail of cysts  $\times 300$



FIG. 8—18 day chick embryonic liver  
4 days  $\times 66$



FIG. 9—18 day chick embryonic liver 4 days  
Detail of liver celled cysts  $\times 320$

All sections stained with Ehrlich's hematoxylin and eosin



## CHORIO ATLANTIC LIVER GRAFTS

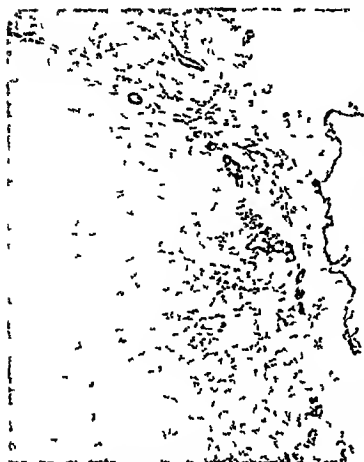


FIG. 6—18 day chick embryonic liver  
3 days  $\times 60$



FIG. 7—18 day chick embryonic liver 3 days  
Detail of cysts  $\times 300$



FIG. 8—18 day chick embryonic liver  
4 days  $\times 66$



FIG. 9—18 day chick embryonic liver 4 days  
Detail of liver colled cysts  $\times 320$

All sections stained with Ehrlich's hematoxylin and eosin



## CHORIO ALLANTOIC LIVER GRAFTS



FIG 10—18-day chick embryo  
liver 6 days Large bile du  
no growth of liver  $\times 66$

FIG 11—18 day chick embryonic  
liver 8 days Large cystic  
bile duct  $\times 66$

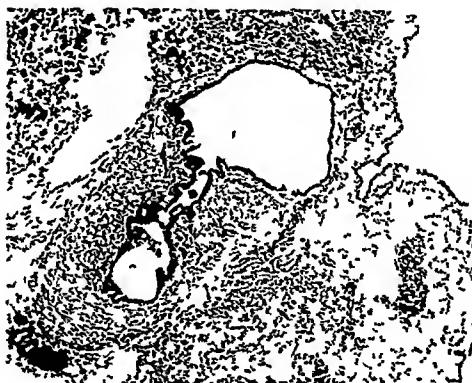


FIG 12—18 day chick embryonic  
liver 8 days Liver cells and  
small bile ducts  $\times 66$





## CHORIO ALLANTOIC LIVER CRAFTS



FIG 13—18 day chick embryonic liver 10 days Liver cells small and large bile ducts, granulation tissue and calcification  $\times 66$



FIG 14—18 day chick embryonic liver 7 days Necrosis of most of transplant with peripheral calcification 66

All sections stained with Ehrlich's hematoxylin and eosin



On the fourth day and after, the trabeculae of liver cells are separated by well developed sinusoids lined by Kupffer cells. Occasionally (especially from the sixth day) large bile ducts appear. They are strikingly different from the liver cells, showing a high columnar epithelium surrounded by a circle of smooth muscle (fig 10). They also may become cystic (fig 11), but retain at least in part their high columnar epithelium with elongated nuclei. No connection between liver cells and these large bile ducts could be made out, they were occasionally present in grafts showing no liver cells. From now on regular growth of liver cells occurs, replacing the necrosed tissue of the transplant if it is not too large. Either several separated nodules of liver occur, or the transplant is replaced by liver cells developed in typical trabeculae and separated by sinusoid-like capillaries (fig 12).

At the 10th or 11th day, beyond which incubation cannot be prolonged owing to the hatching of the chick, the transplant consists of a central necrosed, patchily calcified mass surrounded by a broad band of liver cells, mostly in well formed trabeculae with sinusoids, liver-celled cysts, a few bile ducts, large and small, and a varying but always small amount of granulation tissue (fig 13). Only on one occasion has any connection between bile ducts and liver cells been demonstrated.

Though success was obtained by Danchakoff (1924), Sandstrom and G. L. Kauer (1932) and Van Camperhout and Simard (1932) with other tissues, serial transplants of liver from egg to egg invariably failed, as the granulation tissue always overgrew the liver.

#### *10-day embryo chick liver grafts*

The graft material is very similar to 18-day embryonic chick liver, but contains no demonstrable bile ducts and no fat. Development follows the same lines as that of 17- to 18-day chick embryo liver. The differences are (1) the growth of liver cells, which sometimes contain a little green pigment, is a little better, (2) the great bulk of the transplant is composed of cysts, possibly developed from bile ducts, appearing about the fourth day, by the 8th day the cysts may contain altogether as much as 1 c.c. of fluid, which is yellow, contains much protein and gives a negative direct and indirect van den Bergh reaction. Few or no ordinary bile ducts are present.

#### *Adult hen liver*

Murphy (1914b) stated that grafts of adult hen liver usually produced marked necrosis in the chorio allantois and that if any liver survived it was in the form of a few scattered bile capillaries (1 bile duct). He also found (1916) that mushes of adult liver blown on to the chorio allantois survived grafting. Wilber (1924), comparing the growth of grafts of other organs with that of thyroid grafts, found that liver grew in the chorio allantois.

Grafts were carried out with adult hen liver, age about 2 years. In general the course of events was the same as with embryonic liver, but growth of liver cells, except in one case where it was as good as in embryonic chick liver, was rather poor. Bile ducts were relatively much more prominent and were usually surrounded by a dense circle of fibrous tissue suggesting a portal canal. It is very difficult to obtain adult hen liver certainly free from organisms, and possibly the reaction of the membrane to these transplants may have been due to infection, it was consistently more severe than that of 10- or 17-day embryonic liver.

## II HETEROPLASTIC TRANSPLANTS

Murphy (1913, 1914*b*) showed that tumours from rat and man would grow in the chorio-allantois. This work has been confirmed and extended by Stevenson (1917*a* and *b*, 1918) for the rat and mouse and by Schrek and Avery (1937) for the rat and rabbit.

Hirawa (1927), Hirawa and Wilher (1927) and Nicholas and Rudnick (1932) found poor differentiation and organisation of pieces of 11-day rat foetuses implanted in the chick chorio-allantois.

C. J. Sandstrom and his co-workers (Sandstrom, 1929-30, 1932*a* and *b*, 1933 and 1934, and Sandstrom and J. T. Kauer, 1932, 1933) have carried out an extensive series of transplants of chick kidney on the chorio-allantois of the duck and vice versa. Sandstrom (1929-30) found that duck kidney would grow on the chorio-allantois of the chick until its total age was about 24 days. By the 27th day necroses appeared associated with granulation tissue infiltration. Since at 24 days, duck kidney is fully differentiated, he concluded that "specificity" develops at this time and that the reaction of the membrane is due to this. Later (1932*a*), finding that the reaction to duck tissue was not more severe than that to chick kidney in the chick chorio-allantois, he decided that homoeospecificity was almost as marked in the chick as heterospecificity. Further experiments by Sandstrom and J. T. Kauer (1932, 1933) on kidney and by Sandstrom and G. L. Kauer (1932, 1933) on cartilage and bone transplants convinced him that if reasonable vascularisation is assured (in which the density of the transplanted material, or perhaps its specific character, may play some part (Sandstrom, 1933, 1934)) survival and growth depend on the establishment of function. He contrasts the general failure of grafts of adult kidney, which are never able to build up a secreting mechanism, with, firstly, the active growth of young secreting metanephros (*cf.* Atterbury, 1922-23) and, secondly, with the excellent growth of "passive non-functioning" structures such as cartilage and bone. His final conclusion seems to be that survival of a graft in the chorio-allantois depends entirely on the nature of the transplanted material and not at all on the host.

As grafts, liver from duck embryos of 23 days' incubation and from rat foetuses of about 17 days' gestation was used.

*23-day duck liver.* Almost all the transplants die in the first three days, though even in these mitochondrial stains show a few surviving liver cells scattered round the edge. Of six transplants more than 8 days old, two only showed signs of growth, one of bile ducts only with a surrounding zone of fibrous tissue, the other



to a degree rendering impossible the development of even embryonic tissue of the same species. Moreover though embryonic chick liver grows readily in the chorio-allantois, adult hen liver is sufficiently individualised to grow only poorly, while duck and rat liver (even embryonic) have sufficient specific individuality to grow even less. Poorly individualised structures such as tumours grow readily in the chorio-allantois, but will not grow in the hatched chick (Murphy, 1913).

An attempt was made by Murphy (1914*a*) to demonstrate that a refractory phase occurs in the chorio-allantois, whereby its development of individuality prevents the development of grafts beyond the 20th day of incubation of the host. As Sandstrom (1932*a*) has pointed out, the circulation through the chorio-allantois is at that time degenerating (*cf* Byerly, 1930, on the weight of the allantois), and the "refractory phase" may be only an expression of the poor blood supply of the transplant. Sandstrom's own opinions have changed a good deal with time (see p 114), no doubt all the factors he mentions—vascularisation, differentiation of the graft, function, specificity—play a part. Clearly differentiation cannot be the whole story, for adult hen liver, which must have differentiated completely years before, occasionally grows well in the chorio-allantois. I favour very strongly the view that the chorio-allantois is a poorly individualised structure, which will allow the continued growth of almost any tissue whose degree of individualisation, personal or specific, is not too extreme.

The formation of liver-celled and bile duct cysts makes it clear that both types of cell are secreting, though only in transplants of 10-day liver has any pigment been demonstrated (*cf* Lynch's observations (1921) on tissue culture of liver). The degeneration of transplants has been ascribed by Holton to a change in the metabolic habits of liver cells and by Dalton (1936-37) to retention of secretion. Holton's view is subject to the objection that the change usually occurred when the chorio-allantoic circulation was failing. even if it is true it will not explain the growth of 18-day embryonic liver or adult liver, unless, once the change, *eg* from protein to fat and glycogen as energy sources, has been surmounted, the capacity for growth is regained. Certainly it would be expected that at a time when changes in metabolic methods were occurring the organ would be more vulnerable. However, very few of my transplants show much degeneration of liver cells, and it is very probable that no direct comparison can be drawn between them and livers of chicks of 3-6 days' incubation. No glycogen could be demonstrated in my transplants.

In Cameron and Oakley's autoplasmic transplants of rat liver, bile ducts and liver cells were in the early stages fairly comparable in quantity, while later the liver cells degenerated and disappeared,

leaving only isolated nests of bile ducts. By comparison, transplants in the chorio-allantois—setting aside the grafts of 10-day embryonic liver, which contain hardly any bile ducts—show in almost all cases a marked predominance of liver cells. In the first twenty-four hours, in fact, no bile ducts can be detected. The band of surviving liver cells is in all cases notably thicker on the surface exposed to the artificial sac than it is on that opposed to the chorio-allantois. This rather paradoxical observation may probably be explained by the fact that the chorio-allantois is a respiratory organ whose function is to remove oxygen from the air sac and convey it to the chick, consequently the oxygen supply available by diffusion from the artificial air sac may be greater than that supplied by the chorio-allantois. Possibly the poor results obtained by earlier workers may be in part due to the fact that they used the closed shell method, without artificial air sac. By the third day the transplant is usually entirely incorporated and the difference disappears.

It seems certain that the degree of growth exhibited by liver cells and bile ducts depends not merely on the inherent difference in their capacity for survival but also on the local conditions. Usually bile ducts appear the harder, for they survive to a much greater extent than liver cells in autoplasmic transplants in the omentum (Cameron and Oakley), in the anterior chamber of the eye (Böck and Popper, 1937), in autoplasmic transplants of liver damaged by biliary obstruction (Cameron, 1935), in infarcts of the liver (Cameron and Mayes, 1930) and in tissue culture\* of adult liver (Akamatsu, 1922-23, Mitsuda, 1923, 1924, Börner and Herzog,† 1928-29). In chorio-allantoic grafts however, liver cells develop much more actively.

That local conditions affect the proportional growth of tissues and their degree of differentiation can be illustrated by numerous other examples. Danchakoff (1922) showed that grafts of pronephric primordium in the chorio-allantois invariably showed far greater development of pronephros than over occurred in the developing chick.

Willier and Rawles (1930, 1931a) and Hunt (1931) have shown that liver does not develop in grafts of early chick blastoderms unless heart is present also, though heart often develops without liver. In this connection it is interesting to note Kapel's (1926,

\* Most attempts to grow embryonic liver in tissue culture have been made with liver at a stage of development (3-6 days) when few or no bile ducts are present (Lynch, 1921; Kapel 1926, 1929; Doljanski, 1931), and have consequently thrown little light on the matter, but Dobrowolsky (1916) apparently grew ray liver cells in coagulated ray plasma (species not stated) without any growth of bile ducts.

† Börner and Herzog's claim that transition forms between bile duct epithelium and liver cells can be seen in tissue culture of liver is almost certainly explicable by degenerative changes in the explant, since such transitions are limited to the explant and do not occur in the growing area.



1929) observation that liver grows better in tissue culture if heart is present also. Before plasma was used in tissue culture, cultures of liver usually contained very little liver and large quantities of wandering cells. Akamatsu was the first to show that growth of liver cells could be greatly increased by the use of plasma. This was confirmed by Nordmann (1929). Most important, Doljanski showed that the rate of growth of cells in tissue cultures of liver could be greatly modified by varying the composition of the medium, with results quite different from those which would be expected from the capacity of the cells to survive *in vivo*. If equal quantities of plasma and unheated embryo extract were used, the main growth consisted of fibroblasts and wandering cells, if the embryo extract were heated to 70° C for half-an-hour or added in small amounts, the tissue culture consisted predominantly of liver cells. In the discussion on Doljanski's paper Demuth said that Katzenstein (unpublished observations) had shown that by the addition of cholesterol (1:20,000) to tissue culture media growth of epithelium from liver, kidney and intestine could be greatly favoured.

Finally Fischer and Parker (1929) have shown that cultures of chondroblasts vary in their behaviour according to the composition of the medium. If embryo extract is added to the medium, free growth without differentiation occurs, if the embryo extract is omitted growth is slowed and cartilage and bone are laid down in the explant.

In chorio-allantoic transplants of 3- to 6-day liver where no bile ducts are present liver cells grow freely, in older liver (10-18 days) and in adult liver, as well as in heteroplastic transplants, growth of liver cells is independent of and usually dissociated from that of bile ducts.

#### SUMMARY.

1 Embryonic chick liver, adult hen liver, embryonic duck liver and foetal rat liver grow in the chorio-allantois of the chick.

2 The degree of growth and of reaction to the graft depend almost entirely on the degree of individualisation of the transplanted material.

3 Growth consists almost entirely of liver cells. bile ducts are few and usually dissociated from the liver cells, which are trabeculated, show bile capillaries and are separated by sinusoids. No transition from bile ducts to liver cells has been observed. Erythropoietic masses are occasionally present.

4. Both bile ducts and liver cells secrete.

5 Development of liver cells is favoured at the expense of development of bile ducts.

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culture technique a method in which the cellular constituents of the blood were lysed by means of saponin

A sample of white saponin supplied by British Drug Houses Ltd. was used. It consisted of a mixture of glucosides obtained from quillaria bark and contained metallic impurities in the following proportions —lead, less than 1 part per million, iron, 16 parts per million; copper, 4.5 parts per million; zinc, 8 parts per million. The amount required to produce lysis of different concentrations of citrated human blood, diluted in citrate broth, within 30 mins. at room temperature was determined in an experiment the results of which are recorded in table I

TABLE I.

*Lysis of red blood cells by saponin in blood-broth mixtures containing 0.17 per cent sodium citrate.*

Percentage concentration of blood in mixture	Percentage concentration of saponin in mixture					
	0.1	0.05	0.033	0.025	0.0125	0.001
20	C	C	+	—	—	—
16	C	C	AC*	+	—	—
12	C	C	C	AC	—	—
8	C	C	C	C	—	—
4	C	C	C	C	C	AC

C = complete hæmolysis  
 AC = almost complete hæmolysis  
 + = marked but incomplete hæmolysis  
 — = little or no hæmolysis

\* Almost complete hæmolysis becoming complete after standing overnight at room temperature

It will be seen that a concentration of 0.033 per cent of saponin produced rapid and almost complete lysis of the cells in a blood-broth mixture containing 16 per cent of human blood. Lower concentrations of blood were completely lysed by this concentration of saponin; higher concentrations are rarely used in blood cultures. Lower concentrations of saponin were insufficient to produce immediate hæmolysis.

Concentrations of 0.033 per cent of saponin, 0.17 per cent of sodium citrate and 16.7 per cent. of blood were used throughout this work except when otherwise stated. Most of the experiments were done with one strain of *Streptococcus viridans*.

*The effect of saponin on the growth of streptococci in a blood-broth mixture.* Estimations of the growth of streptococci were made using broth with the addition of defibrinated, citrated and saponin-lysed citrated rabbit's blood (table II). This experiment was repeated with consistent results on several occasions with inocula of different sizes and also with a different strain of *Str. viridans*.



It will be seen that both sodium citrate and saponin have an inhibitory effect on the growth of streptococci in broth in these concentrations and that the inhibition is more marked with sodium citrate than with saponin

*Effect of saponin and sodium citrate on the growth of streptococci in nutrient broth containing water-lysed blood* To determine whether the inhibitory effect of the saponin was still active in a blood-broth mixture but masked by the enhanced growth of streptococci due to other changes in the medium resulting from lysis of the cells, the growth of streptococci in broth containing lysed blood and saponin was next compared with that in broth containing defibrinated and citrated blood lysed by distilled water

To one tube containing 16 c c of sterile distilled water were added 4 c c of defibrinated rabbit blood, to a second 4 c c of defibrinated rabbit blood and saponin and to a third 4 c c of citrated blood. Hæmolysis having taken place, 4 c c of broth of four times the normal strength were then added to each tube, thus bringing all constituents to the same concentration as used in the other experiments (table IV).

TABLE IV.

*Effect of saponin and sodium citrate on growth of Str viridans in broth containing water-lysed blood.*

Viable counts at	Broth + water-lysed defibrinated rabbit blood Organisms per c c	Broth + water-lysed defibrinated rabbit blood + saponin Organisms per c c	Broth + water-lysed citrated rabbit blood Organisms per c c
0 hours	9	9	10
1½ "	12	10	10
3 "	11	12	8
4 "	23	25	14
5 "	76	76	32
6 "	176	162	90
9 "	15,500	16,600	4,000
13 "	2,550,000	2,590,000	810,000
16 "	24,200,000	36,000,000	11,600,000
20 "	2,032,000,000	1,920,000,000	1,616,000,000

The addition of saponin will be seen to have no significant effect on the rate of growth of streptococci in a water-laked blood-broth mixture, whereas the addition of sodium citrate has a slight inhibitory effect

*The effect of serum and blood cells on the growth of streptococci in citrate broth* Since lysis of cells enhanced the growth of streptococci in a blood-broth mixture, it was necessary to determine whether the improved growth was due to the removal from the medium of suspended cells. Growth estimations were therefore made in sodium citrate broth to which had been added in one instance the cells, and in another the serum, derived from defibrinated rabbit blood; two further growth estimations were

made in broth containing saponin in addition to cells and serum (table V)

TABLE V

*Growth of Str viridans in citrate broth containing serum and blood cells with and without the addition of saponin*

Table counts at	Citrate broth + serum Organisms per c.c.	Citrate broth + serum + saponin. Organisms per c.c.	Citrate broth + blood cells Organisms per c.c.	Citrate broth + blood cells + saponin Organisms per c.c.
0 hours	6	0	6	6
2 "	10	0	10	12
4 "	25	21	94	98
6 "	50	88	041	597
8 "	104	185	2,010	3,530

It will be seen that growth of streptococci in a blood-broth mixture is not enhanced by the removal of the cellular constituents of the blood

*The effect of the addition of lysed blood cells to a whole blood-broth mixture* The remaining explanation that would account for the enhanced growth of streptococci in a lysed blood-broth mixture appears to be that in lysing the cells, some diffusible product is liberated into the mixture and improves the growth of the organisms. In order to test this possibility, the growth of streptococci was compared in citrate broth containing (a) whole blood, (b) whole blood + water-lysed cells, (c) water-lysed blood and (d) saponin-lysed blood (table VI)

TABLE VI

*Effect of lysed blood on growth of Str viridans*

Table counts at	Citrate broth + whole blood Organisms per c.c.	Citrate broth + whole blood + water lysed cells Organisms per c.c.	Citrate broth + water lysed blood Organisms per c.c.	Citrate broth + saponin lysed blood Organisms per c.c.
0 hours	5	5	5	5
2 "	5	35	5	10
4 "	10	25	40	15
6 "	175	202	391	133
8 "	400	690	2,135	1,020
12 "	3,420	27,750	132,000	114,000

The results obtained indicate that the enhanced growth of streptococci in a blood-broth mixture occurs when lysed and whole blood are present together and it is therefore probably due to the addition of some diffusible product liberated into the medium on lysis of the blood cells

*Variation in pH of citrate broth containing whole blood, saponin-lysed and distilled water lysed blood* The pH of three types of blood-broth mixture containing (a) whole blood, (b) saponin-lysed



blood and (c) distilled water-lysed blood, was determined by the electrical method, a glass electrode being used in order to prevent reduction. The mixtures were inoculated with streptococci and the pH determined immediately after inoculation, after 4 hours' incubation at 37° C when growth usually commences, and after 24 hours' incubation when growth is complete.

TABLE VII  
*Reaction of blood-broth mixtures*

pH estimations at	Citrate broth + whole blood	Citrate broth + water-lysed blood	Citrate broth + saponin-lysed blood
0 hours	7.28	6.98	7.36
4 "	7.26	7.10	7.46
24 "	6.0	6.0	6.10

*The effects of higher concentrations of saponin on the growth of streptococci in blood-broth mixtures* It will be seen from table I that, by increasing the concentration of saponin above 0.033 per cent, more rapid and complete hæmolysis occurred in broth containing 16 per cent. of blood. The use of such concentrations of saponin was attended by a further enhancement in the growth of streptococci in the lysed blood-broth. That the increased rapidity of growth was due to the more complete hæmolysis was shown by the fact that a similar effect could be produced by delaying the inoculation of blood-broth containing 0.033 per cent. of saponin until the mixture had been incubated for 3 hours at 37° C, by which time the blood was completely lysed, growth then proceeding as rapidly as in blood-broth containing a higher concentration of saponin. The improvement ceased to be apparent with concentrations of saponin greater than 0.5 per cent, although concentrations as high as 5 per cent appeared to have no bactericidal or inhibitory effect on the growth of streptococci in blood-broth as shown by viable plate counts during the first 24 hours of incubation.

*The effect of saponin on the growth of other bacteria in blood-broth mixtures* Using bacteria other than *Str. viridans*, the rate of growth was compared in sodium citrate blood-broth with and without the addition of saponin. *Str. pneumoniae* and *Bact. coli* grew more rapidly in saponin-lysed blood-broth containing saponin up to a concentration of 4 per cent (higher concentrations were not tested). *Str. pyogenes* was itself strongly hæmolytic and showed no marked sign of improved growth when saponin was added to the medium. *Str. faecalis* and *Staph. aureus* appeared to grow equally well in whole and in saponin-lysed blood-broth.

Comparative viable plate counts were rendered difficult in the case of *Staph aureus* owing to clumping of the organisms

**Application of lysed blood technique to blood cultures following dental extraction**

*Comparison of results obtained using whole and lysed blood in parallel in blood cultures taken from 65 patients immediately following the extraction of teeth* Ten c c of blood were taken from each patient immediately following operation. Half of this was cultivated in the original manner using whole citrated blood, the remainder was added to 0.6 c c of a solution of saponin in sodium citrate saline and finally distributed into agar and citrate broth as before, giving a final concentration of 0.17 per cent sodium citrate and 0.033 per cent saponin. All cultures were incubated aerobically only and standard transplants made from the broth tubes daily for 7 days into nutrient broth. When growth occurred in these transplants it was plated on 5 per cent horse blood agar for identification.

Of the 65 patients investigated, 39 were found to have positive blood cultures, *Str viridans* being recovered in each instance. Of these 39 cultures, 38 or  $97.4 \pm 2.6$  per cent were positive using the lysed blood technique and 27 or  $71.1 \pm 7.3$  per cent using the whole blood method. The percentage difference (26.3 per cent) is greater than three times the standard error of the difference (7.8 per cent).

Details of the findings and the types of cases examined are recorded in table VIII. Of 20 whole blood cultures 14 appeared after 24 hours, 2 after 2 days, 3 after 3 days and one after 7 days, the corresponding saponin-treated specimens gave 15 cultures after 24 hours, 3 after 2 days and 2 after 3 days.

**TABLE VIII**  
*Comparison of whole blood and saponin methods in blood cultures after dental extraction*

Type of case	Cases examined	Results of blood cultures after extraction of teeth		Streptococcal colony count.									
				< 1 per c.c.		1-5 per c.c.		6-10 per c.c.		11-15 per c.c.		> 15 per c.c.	
		Whole blood	Lysed blood	W	B	W	B	W	B	W	B	W	B
Gum disease moderate or marked, multiple extraction	20	10+ (=50%)	14+ (=70%)	8	8	1	5	1	-	-	-	-	1
Without detectible gum disease extraction of one or more teeth	45	17+ (=37.8%) 23-	23+ (=51.1%) 21-	0	14	7	7	-	2	1	-	-	1

W B = whole blood

L B = saponin lysed blood

+ = streptococci present in the blood cultures

- = no streptococci present in the blood cultures

shown, moreover, that the nature of the diluent is of importance in determining the growth of organisms, sodium citrate broth being superior to distilled water in this respect (Wright), the addition of concentrated broth to the blood after lysing the cells with water would necessitate an additional manipulation with an increased risk of contamination

Saponin in a concentration of 0.033 per cent produces rapid lysis of both red and white cells in a citrate blood-broth mixture containing 16 per cent of blood and 0.17 per cent of sodium citrate, lower concentrations of blood require less saponin to produce the same effect. Saponin has an additional advantage as a lytic agent in that it may be incorporated in the broth to be used as diluent before autoclaving.

The advantage of using saponin-lysed blood as compared with whole blood in attempting to recover streptococci from blood cultures has been demonstrated in blood cultures from 65 patients immediately following the extraction of teeth. From citrated whole blood streptococci were recovered in 27 cultures or 71.1 per cent of positive cases, whereas from saponin-lysed blood streptococci were recovered in 38 cultures or 97.4 per cent of positive cases, 39 of the 65 patients having a demonstrable positive blood culture following operation. In only one case were streptococci isolated from the whole blood and not from the lysed blood culture.

Four cases of subacute bacterial endocarditis are recorded, in two of which attempts to grow the causative organism in whole citrated blood failed, whereas a *Str. viridans* was recovered from the saponin-lysed blood after two days' incubation. It is noteworthy that streptococci could be isolated from the whole blood samples during the first few hours of incubation by removing 1 c.c. quantities from the blood and plating in agar. From one other case a *Str. viridans* was isolated, the organisms growing in the lysed blood sample after 10 hours, whereas in the whole blood no growth occurred until after 3 days' incubation. From the fourth case a *Str. faecalis* was recovered which grew equally well and without a prolonged lag phase of growth in both whole and lysed blood.

### Summary

1. Blood cultures may be unreliable when whole blood is used and few organisms are present.
2. Low concentrations of saponin lyse both red and white blood cells.
3. The growth of *Str. viridans* and certain other organisms is enhanced by the addition of saponin to sodium citrate blood-broth.
4. Using whole blood and saponin-lysed blood in parallel, streptococci were recovered in 27 whole blood samples and 38

lysed blood samples out of a total of 39 positive blood cultures from 65 patients following dental extractions

5 In 2 cases of subacute bacterial endocarditis a *Str viridans* grew in the saponin-lysed blood but failed to grow in the whole blood samples From the third case a *Str viridans* was recovered which grew earlier in lysed than in whole blood, while in the fourth case, from which a *Str faecalis* was isolated, no advantage was gained by the use of saponin

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It was subsequently found that the undiluted saponin-lysed blood before distribution into broth and agar was markedly bactericidal for streptococci. The technique was therefore modified so that the necessary amount of saponin was incorporated in the citrate broth to which the blood was then added direct from the syringe. In the event of a viable count being required, suitable quantities of the lysed blood-broth mixture were plated in agar. During the performance of the 65 blood cultures described above an unavoidable delay of from half-an-hour to 2 hours elapsed between the time at which the blood was taken from the patient and its subsequent inoculation into broth. It is probable, therefore, that some of the negative results obtained in the lysed samples were due to the toxic action of the high initial concentration of saponin in the undiluted blood.

### Application of lysed blood method to blood cultures in subacute bacterial endocarditis.

*Influence of saponin on the bactericidal effect of blood and on the rate of growth of streptococci in blood cultures.* It has been shown by Wright that in many cases of subacute endocarditis the blood has bactericidal properties for the infecting organism. In four cases a comparison was made between the bactericidal effect of whole blood and blood lysed by saponin.

Blood was withdrawn from an arm vein and 4 c c samples were inoculated into 20 c c quantities of citrate broth and saponin citrate broth and incubated at 37° C. Two 1 c c quantities were withdrawn from each at stated intervals and plated in 10 c c agar, the number of viable organisms being estimated by counting the colonies produced in the plates after 48 hours' incubation at 37° C (table IX).

TABLE IX.

*Effect of lysis by saponin on destructive action of blood from cases of infective endocarditis upon streptococci in broth*

Case and infecting organism	State of blood in broth	Streptococcal colonies developing from 1 c c blood-broth mixture after incubation at 37° C for stated period											
		0 hr	1 hr	2 hrs	4 hrs	8 hrs	12 hrs	24 hrs	30 hrs	48 hrs	72 hrs	7 days	14 days
1. <i>Str. viridans</i>	Whole	19.5			1.5	—	—	—	—	—	—	—	—
	Lysed	18.5			11.0	2.0	1.0	1.0	—	+	—	—	—
2. <i>Str. viridans</i>	Whole	11.0	7.0	7.0	3.5	3.0	1.5	2.5	2.5	1.5	+		
	Lysed	6.0	6.0	5.0	8.0	48.0	+						
3. <i>Str. viridans</i>	Whole	2.0	1.0	1.0	1.0	1.5	—	—	2.5	4.0	—	—	—
	Lysed	0.5	3.5	1.0	1.5	0.5	—	0.5	10.0	111.0	+		
4. <i>Str. faecalis</i>	Whole	3.5	6.5	10.5	153.5	+							
	Lysed	8.5	9.0	13.5	256.0	+							

+ = too many colonies to count

In all four cases cultures developed from the lysed blood whereas no growth occurred in two from the whole blood although

there were organisms present at the beginning of the experiment. The blood culture from case 1 was repeated but, as on the previous occasion, no growth occurred after 14 days' incubation of the whole blood, whereas growth occurred in the lysed blood after 2 days' incubation. Two further cultures made by independent investigators using whole blood yielded negative results.

*Influence of saponin on the rate of growth of streptococci in blood cultures* In many blood cultures from cases of subacute bacterial endocarditis, observable growth of streptococci does not occur until after several days' incubation. It can be seen from table IX, case 2, that growth appeared first in the lysed blood samples.

To 5 cc quantities of citrate broth and saponin-citrate broth were added small quantities of blood from two patients suffering from infective endocarditis (cases 1 and 2, table IX), so that in each case there were five tubes containing whole and five containing lysed blood-broth mixtures. All were incubated at 37° C for 7 days and a standard inoculum taken at 24-hour intervals into nutrient broth and incubated for 48 hours. In the first case, whose blood contained about 19 streptococci per cc, no tube inoculated from the whole blood yielded any growth, whereas from the lysed blood 1 tube yielded a growth on the first day, 4 on the 2nd and 5 on each subsequent day. In the second case (8.5 streptococci per cc) the whole blood gave one culture at the 1st attempt, 4 each at the 2nd to 6th and 5 at the 7th, while the lysed blood gave 4 at the 1st and 5 at all subsequent attempts.

### Discussion

It is probable that methods of blood culture dependent on the addition of citrated whole blood to a nutrient broth are unreliable when only small numbers of organisms are present. This is more especially the case when attempts are made to cultivate organisms from the blood of patients with subacute bacterial endocarditis, since in these cases the infecting organisms commonly exhibit a prolonged "lag" phase of growth, during which period they are exposed to various inimical influences in the blood which are not completely abolished by dilution.

Leucocytes may retain phagocytic properties after incubation for 24 hours in a broth medium such as is used for blood culture purposes. The risk of phagocytosis occurring in blood cultures is avoided by lysing the blood cells. In addition, the growth of certain organisms, notably the *Streptococcus viridans*, is enhanced by lysis of the blood cells in the culture medium.

Water may be used as the lytic agent but I have found that it does not produce such complete lysis of the leucocytes as saponin in the presence of 0.17 per cent sodium citrate. It has been

shown, moreover, that the nature of the diluent is of importance in determining the growth of organisms, sodium citrate broth being superior to distilled water in this respect (Wright), the addition of concentrated broth to the blood after lysing the cells with water would necessitate an additional manipulation with an increased risk of contamination

Saponin in a concentration of 0.033 per cent produces rapid lysis of both red and white cells in a citrate blood-broth mixture containing 16 per cent of blood and 0.17 per cent of sodium citrate, lower concentrations of blood require less saponin to produce the same effect. Saponin has an additional advantage as a lytic agent in that it may be incorporated in the broth to be used as diluent before autoclaving.

The advantage of using saponin-lysed blood as compared with whole blood in attempting to recover streptococci from blood cultures has been demonstrated in blood cultures from 65 patients immediately following the extraction of teeth. From citrated whole blood streptococci were recovered in 27 cultures or 71.1 per cent of positive cases, whereas from saponin-lysed blood streptococci were recovered in 38 cultures or 97.4 per cent of positive cases, 39 of the 65 patients having a demonstrable positive blood culture following operation. In only one case were streptococci isolated from the whole blood and not from the lysed blood culture.

Four cases of subacute bacterial endocarditis are recorded, in two of which attempts to grow the causative organism in whole citrated blood failed, whereas a *Str viridans* was recovered from the saponin-lysed blood after two days' incubation. It is noteworthy that streptococci could be isolated from the whole blood samples during the first few hours of incubation by removing 1 c.c. quantities from the blood and plating in agar. From one other case a *Str viridans* was isolated, the organisms growing in the lysed blood sample after 10 hours, whereas in the whole blood no growth occurred until after 3 days' incubation. From the fourth case a *Str faecalis* was recovered which grew equally well and without a prolonged lag phase of growth in both whole and lysed blood.

### Summary

- 1 Blood cultures may be unreliable when whole blood is used and few organisms are present

- 2 Low concentrations of saponin lyse both red and white blood cells

- 3 The growth of *Str viridans* and certain other organisms is enhanced by the addition of saponin to sodium citrate blood-broth

- 4 Using whole blood and saponin-lysed blood in parallel, streptococci were recovered in 27 whole blood samples and 38

lysed blood samples out of a total of 39 positive blood cultures from 65 patients following dental extractions

5 In 2 cases of subacute bacterial endocarditis a *Str viridans* grew in the saponin-lysed blood but failed to grow in the whole blood samples From the third case a *Str viridans* was recovered which grew earlier in lysed than in whole blood, while in the fourth case, from which a *Str faecalis* was isolated, no advantage was gained by the use of saponin

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## THE GROSS ANATOMY OF THE PARATHYROID GLANDS

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IN the course of previous work in which the weights of parathyroid glands were analysed statistically (Gilmour and Martin, 1937), I removed and examined microscopically the glands in a consecutive unselected series of 428 necropsies. This series is used here for a study of the gross anatomy of these glands. Reference is also made to conditions of interest found in subsequent dissections. The observations and the literature cited refer to man except when otherwise stated.

### Number

In 428 dissections 1713 glands were found, an average of 4 in each subject

2 glands were found in	1 case	(0.2 per cent)
3 " " " "	26 cases	(6.1 " )
4 " " " "	374 "	(87 " )
5 " " " "	25 "	(6 " )
6 " " " "	2 "	(0.5 " )

The number of glands given in this list depends upon microscopic examination. Very rarely two glands were bound together in a single capsule so as to look like one to the naked eye, a condition which has been described by Welsh (1897-98), Erdheim (1907) and Yanaso (1908).

When only 2 or 3 glands were found in this series their relatively small weight suggested that all the glands had not been found (Gilmour and Martin). Further, in a study of 16 embryos and fetuses at least 4 glands were found in 14 in which the whole parathyroid-bearing area had been examined in serial microscopic sections (Gilmour, 1937). In a very few recent dissections, however, I have failed to find more than 2 or 3 glands, and those found were not exceptionally small. Moreover, in serial microscopic sections of the neck Rössle (1932) found no parathyroid tissue in an infant aged 9 hours and only one doubtful structure on the left side in an infant of 10 weeks, while Böttiger and Wernstedt

(1927) found in an infant of 4 months only one parathyroid which lay upon the lower part of the right lobe of the thyroid. The possibility, therefore, of a real diminution in number below 4 must be recognised.

When 5 or 6 glands were found they were occasionally all of about the same size. As a rule, however, the supernumerary glands were small and were considered to be accessory glands. The accessory glands often lay close to a gland of normal size but sometimes at a little distance from it. Very small glandular nodules, about 1 mm. in diameter, were occasionally seen close to but free from glands of normal size, these were not accepted as accessory glands. Origin from pharyngeal pouches III or IV could be assigned to 27 of the 29 supernumerary glands (see below, pp 138-140). 13 of these 27 were parathyroids III (6 right, 7 left) and 14 were parathyroids IV (10 right, 4 left).

Six was the maximum number of glands in any one case in this series. In the 14 embryos and fetuses in which the whole parathyroid-bearing area had been examined in serial microscopic sections in my embryological study 6 glands were again the maximum found. 4 glands were found in all fourteen, 2 accessory glands were found in one subject, 1 accessory gland and 1 germ of an accessory gland in another and 1 accessory gland in a third (Gilmour). Further, in all the dissections which I have made in addition to the 128 under analysis I have never found more than 6 parathyroid glands.

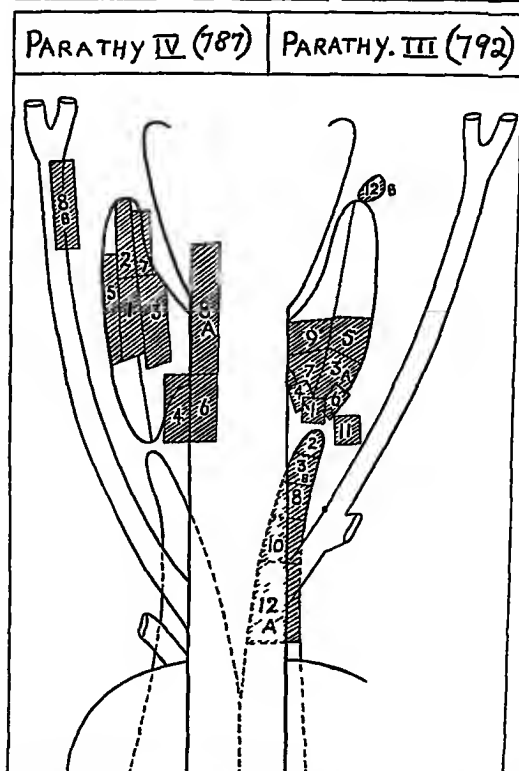
Erdheim (1904a) described an infant with 12 glands, 8 being very small and 4 being large, and another infant with 8 glands, 4 being very small and 4 being large. His observations are open to criticism because the accessory glands were very small and merely germs of parathyroids. It is indeed possible that these accessory glands of Erdheim were the buds of parathyroids which led to the formation of what I have termed Kurlemeyer's type 1 (Gilmour). Zuckersandl (1902) mentioned the presence of supernumerary glands, but he did not say whether their parathyroid nature could be proved. Erdheim (1904b) also, and said he only mentioned them in a footnote. Papanicolaou (1907, 1907b) described 11 glands in an infant of 4 months, but he is probably that he had identified them as parathyroids. He makes the remarkable statement that the parathyroid glands are numerous and that the greatest number ever counted was 100. Papanicolaou (1907b) described the parathyroid glands as consisting of parathyroid cells about a gland. It is not clear, however, that he intended to describe the parathyroid glands as being situated within an adipose capsule. Erdheim (1904b) also states that the parathyroid glands are situated within an adipose capsule. Zuckersandl (1902) also states that the parathyroid glands are situated within an adipose capsule. Erdheim (1904b) also states that the parathyroid glands are situated within an adipose capsule. Zuckersandl (1902) also states that the parathyroid glands are situated within an adipose capsule.

### Parathyroid

The embryology of the parathyroid gland is described in 1927, and 1928, on each side the

third pharyngeal pouch gives rise to a parathyroid gland (parathyroid III) and one lobe of the main and constant thymus (thymus III), while the fourth pharyngeal pouch gives rise to a parathyroid gland (parathyroid IV) and an inconstant separate

### POSITIONS OF PARATHYROID GLANDS



The numbers indicate the sites occupied by parathyroids III and IV in order of decreasing frequency, as detailed in the text. The line drawn down each lobe of the thyroid represents the posterior border, which has been retracted laterally.

portion of thymus (thymus IV). The different positions occupied by parathyroids III and IV in the present series and the relative frequency with which these positions were occupied were worked out, and are shown in the accompanying figure. The criteria by

which the pouch of origin was ascertained can be explained more easily after the sites and their relative frequency have been described

### Parathyroids III.

The positions of 792 parathyroids III were arranged in 14 groups, which are numbered in the text below and in the figure in order of diminishing frequency of occurrence

1. On the thyroid at or just behind its lower pole, or not more than 0.5 cm. below the lower pole. Number found in this position. 127 (53.9 per cent.)

2. One cm. below the lower pole of the thyroid. 101 (12.8 per cent.)

3A. On the posterior part of the outer surface of the thyroid 1-1.5 cm. above its lower pole. 55 (6.9 per cent.)

3B. Two cm. below the lower pole of the thyroid. 55 (6.9 per cent.).

4. On the thyroid just internal to its lower pole, between the thyroid and the trachea. 51 (6.4 per cent.)

5. On the posterior part of the outer surface of the thyroid 1.5-2.5 cm. above its lower pole. 41 (5.2 per cent.)

6. On the thyroid just external to its lower pole. 18 (2.3 per cent.).

7. Upon the mesial surface of the thyroid 1-1.5 cm. above its lower pole between the thyroid and the trachea. 13 (1.6 per cent.).

8. Three cm. below the lower pole of the thyroid: 11 (1.4 per cent.).

9. On the mesial surface of the thyroid 1.5-2.5 cm. above the lower pole, between the thyroid and the trachea. 9 (1.1 per cent.).

10. Four cm. below the lower pole of the thyroid: 7 (0.9 per cent.)

11. Attached to the sterno-thyroid muscle below and in front of the lower pole of the thyroid. 2 (0.3 per cent.)

12A. Six cm. below the thyroid. 1 (0.1 per cent.).

12B. At the upper pole of the thyroid. 1 (0.1 per cent.).

Only one of the glands (204/34) was found on the anterior surface of the thyroid. It lay just in front of the lower pole and has been included in position 1.

The glands below the thyroid are figured as if they lay on thymus III. They actually lay either in it or in involuted remnants of it or within separate accessory portions of it known as "thymus lobules III," or in corresponding positions. The relative numbers found in these different relations are given on p. 142.

*More recent dissections* have revealed a few other unusual positions of parathyroids III.

(1) In one subject (178/36) the right parathyroid III lay in the

upper pole of the thymus, which extended to a point 1.5 cm below the bifurcation of the common carotid artery and just above the upper pole of the thyroid. It lay 3 cm above the right parathyroid IV.

(2) In another subject (38/30) the right parathyroid III was within the upper of two discrete "thymus lobules III". These lay one above the other above the main thymus III, which ended on that side 1 cm above the left innominate vein. The gland lay close to the carotid artery and was at the same level as the parathyroid IV, which lay near the centre of the posterior border of the thyroid (position IV 1).

(3) In one subject (73/37) the right and left parathyroids III lay deep within the thymus, one 0 cm the other 11 cm below the level of the lower poles of the thyroid. The lower lay over the pericardium opposite the upper level of the ventricles. This is the lowest gland that I have so far found. Bergstrand (1934) described a parathyroid tumour in the thymus over the pericardium in a case of generalised osteitis fibrosa, 4 normal glands being found in the usual positions.

#### Parathyroids IV

The positions of 787 parathyroids IV could in a similar manner be arranged in 9 groups

1 On the middle third of the posterior border of the thyroid, the upper part of this area being the site of election. The gland often lies in a groove in the thyroid or on a projecting nodule, the thyroid tubercle of Zuckerhändler, which is frequently present in this region. The gland usually lies above the inferior thyroid artery, but is sometimes behind the artery or between it and the thyroid or wrapped round it, as described by Von Verebely (1907). It is rarely below the artery. Number found in this position 580 (73.7 per cent.)

2 On or close to the upper third of the posterior border of the thyroid 74 (9.4 per cent.)

3 On the mesial surface of the lateral lobe of the thyroid opposite the middle third of its posterior border, where vessels frequently enter—a position described by Kursteiner (1898-00) and Benjamins (1902) 57 (7.24 per cent.)

4 Between the lower third of the posterior border of the thyroid and the oesophagus, in a somewhat more posterior plane than that of the lower pole of the thyroid and in the same plane as the oesophagus 46 (5.85 per cent.)

5 On the thyroid near the centre of the posterior half of the outer surface of the lateral lobe 12 (1.52 per cent.)

6 Behind the oesophagus, opposite the lower pole of the thyroid or the lower third of its posterior border 0 (1.14 per cent.)

7. On the mesial surface of the lateral lobe of the thyroid opposite the upper third of its posterior border · 5 (0·63 per cent.)

8A. Behind the œsophagus or pharynx opposite the upper two-thirds of the posterior border of the thyroid · 2 (0·25 per cent.).

8B. In the carotid sheath lying within a discrete pad of fat on the inner side of the common carotid artery 2·3 cm below its bifurcation · 2 (0·25 per cent.)

*In more recent dissections* there were two cases (23/36, 97/36) in which the parathyroids IV lay in pads of fat between the thyroid and carotid artery in a position slightly below that of IV 8B

Schäper (1895) found in a new-born infant 2 small glands together just below the bifurcation of the carotid. Two other glands were found on each side behind the thyroid, so that it is not possible to determine whether the supernumerary glands came from pouch III or pouch IV.

As in the case of parathyroids III there was only one parathyroid IV that lay anterior to the thyroid. A parathyroid IV lay just in front of the upper pole (438/34). It has been included in position IV 2

Sandström (1880) and von Verheyl (1907) described a gland in front of the lower pole of the thyroid. Millner (1927, 1931) said that glands occur with high frequency on the anterior surface of the thyroid

### *Determination of the pouch of origin.*

One or more of the following criteria, which all depend upon embryological observations, were used to determine the pharyngeal pouch of origin of the parathyroids in any given case. (a) The close association between the parathyroid and the thymus derived from one or other pouch. During development parathyroid III forms a head to thymus III which extends caudally from it. At its attachment thymus III becomes drawn out into a neck. The neck usually breaks so that the two structures separate but it may remain and develop in bulk. Parathyroid IV in early stages has similar relations to thymus IV. (b) The positions which the parathyroid and associated thymus characteristically come finally to occupy during development. (c) The fact that during embryological development parathyroid III usually comes to occupy a lower position than parathyroid IV. Because of the more rapid growth of the neck of the embryo than of thymus III and the greater development in bulk of the caudal part of thymus III the thymus descends relatively to the thyroid and drags the attached parathyroid III with it. As can be seen from the figure there are exceptions to this usual course of development. Consequently the fact of two glands can only be accepted as a parathyroid III pouch if there is no evidence to the contrary or there is positive





sulated, sometimes spindle-shaped pads of fat, in which small portions of thymic epithelium were occasionally found. There can be little doubt that such lobules of fat in these positions represent vestigial remnants of thymus IV. The remaining positions, IV 3, 5, 2, 8B, are not obviously associated with thymus IV. The parathyroids in these positions which were judged to be parathyroids IV were so judged because there was evidence that the other parathyroid on that side was a parathyroid III or because they occupied higher positions than the other parathyroid, which lay in a position compatible with that of a parathyroid III.

The finding of yet other parathyroids IV in close relation to the common carotid artery in position 8B in two recent dissections is of interest, because all authors state that during embryological development parathyroid IV and its associated thymus IV (ultimobranchial body) always migrate to the posterior border of the thyroid.

Neither in the present series nor in more recent dissections has a parathyroid been found at the bifurcation of the common carotid artery, and there is no record in the literature of such a finding in man. It is difficult to understand this, because in human embryology parathyroid III starts above the bifurcation while in certain animals the bifurcation is the usual position of parathyroid III.

It will be seen that 134 of the 1713 parathyroids were not employed in grouping the positions of parathyroids III and IV. Single glands found on one side were omitted. In five cases 2 glands on one side lay so close together that it was not possible to determine which was parathyroid III and which IV. More numerous omissions were due to the notes on position being found to be insufficiently precise.

*The relation between parathyroids III and IV and the upper and lower parathyroids*

In the normal course of development, as explained above, parathyroids III become the lower parathyroids. The figure shows that many of the positions of parathyroids III and IV are on the same level. This suggests that there are many exceptions to the normal relation of the glands. Actually in the whole series of 428 cases investigated there were the 5 cases in which the two glands on one side lay so close together that it was impossible to tell which was parathyroid III and which IV. Here neither gland was lower than the other. Further, among the 1579 glands which were determined to be parathyroids III or IV there were only two cases in which upper glands were undoubtedly para-

thyroids III In one case parathyroid III on one side was attached to an abnormal thymus III and, occupying the exceptional position III 12B, was higher than parathyroid IV In another case the parathyroid III occupied position III 2 and was slightly higher than the parathyroid IV in position IV 6 It is true that in certain instances the pouch of origin of the parathyroids was determined solely by their relative position so that the question was begged, but the number of these was very small In spite, therefore, of the overlapping of the positions in which parathyroids III and IV are found it can be concluded that there are very few exceptions to the rule that parathyroid III becomes the lower and parathyroid IV the upper gland

Among the unusual positions of parathyroids III revealed by later dissections and given on pp 136 and 137, there are two other instances of exception to the rule In case (1) the right parathyroid III lay 3 cm above the right parathyroid IV In case (2) parathyroids III and IV lay at the same level

#### *Relationship to other tissues*

*Fat* The glands frequently lie among lobules of adipose tissue from which they are easily separated Both parathyroids III and IV sometimes lie, however, within discrete pads of fat from which they can only be removed by incision These fatty pads are of rounded, sausage or spindle shape They usually have a delicate capsule and are not subdivided into smaller lobules like ordinary adipose tissue, so that they tend to be clearly defined from other tissues Occasionally they are greyer than ordinary fat A gland inside such a pad is usually visible externally as a brownish area Thymic tissue was found in a few of such pads as were examined microscopically These pads appear to be involuted thymic glands, either thymus III, thymus lobules III or thymus IV The frequency with which parathyroids IV in positions 8A, 4 and 6 were found in these pads has already been mentioned Pads were also relatively common in positions III 2, 3B and 8 But glands within such pads were found also in all the other positions of parathyroids III and IV except III 12A, 10, 11 and 12B It has already been mentioned that the two parathyroids IV in the carotid sheath in position 8B, and the parathyroids IV found in a slightly lower position than 8B in two more recent dissections, all lay in such pads

Kursteiner (1898-99), Benjamins (1902), Getzowa (1907) and Fischer (1911) described the occurrence of parathyroids in discrete masses of fat

*Thyroid* The majority of both parathyroids III and IV lay upon the thyroid Occasionally they were included within the capsule or lay beneath it, or, especially in goitres, projected from

a sulcus between two lobules of thyroid. They were sometimes carried partially into the thyroid by vessels. Position 3 for parathyroids IV was the commonest site in which glands lay under the capsule or were carried partially into the thyroid by vessels. Very rarely parathyroid and thyroid tissues were found on microscopic examination to be fused, without any intervening fibrous septum. In the unselected series no parathyroids were completely covered by thyroid tissue. In two other dissections (71/36, 238/26) however, a gland was completely within the thyroid, one being in the centre of a lateral lobe. Both these internal glands were apparently parathyroids III, because glands were found at a higher level on the same side in the positions of parathyroids IV. They were therefore not strictly comparable with the internal parathyroids of certain animals, which are derived from the fourth pouch. But in one of the fetuses examined in my paper on embryology (Gilmour 1937) a small accessory parathyroid IV lay deep within the thyroid beside a thymus IV.

Kur-tomer (1898-99), in a 9 cm foetus and Pepete (1907-08) described direct continuity between thyroid and parathyroid tissue. Muller (1896), Schröder (1898), Bergstrand (1920), Lahey (1926) and others have described parathyroids within the thyroid and regarded them as homologous with the internal parathyroids IV of certain animals. Iversen, according to Grasmanu (1923), found 6 parathyroids within the thyroid and 12 within its capsule in 96 thyroids cut into thin serial slices.

*Recurrent laryngeal nerve.* Parathyroids IV in position 3 and parathyroids III in positions 7 and 9 were occasionally in contact with this nerve.

*Sternothyroid muscle.* The two glands in position III 11 were attached to the sternothyroid muscle. Voluntary muscle was found microscopically in two cases on the surface of glands in positions III 1 and 2, and probably came from the same muscle.

*Paratracheal lymph glands.* Included in positions III 2 and 3B are parathyroids III which lay alongside the trachea. Here they were in close relation to the lymph glands and could easily be confused with them. Sometimes such parathyroids were contained in the attachment to the trachea of the upper pole of the thymus which was turned inwards.

*Thymus.* In 102 subjects parathyroids III or IV were seen with the naked eye to be within thymic tissue. The parathyroid III in position 12B formed the upper pole of an abnormal upward extension of one lobe of thymus III. Of the 175 parathyroids III 117 (66.8 per cent) were seen with the naked eye to be within thymus III or its involutional remains and 3 (1 per cent) to be within "thymus IV or III." In all therefore 121 parathyroids III were seen or probably to be in thymus III, its involutional remains or

"thymus lobules III" These 121 parathyroids were found in 99 (23 per cent) of the 426 subjects, 44 of the glands within the thymus or its involuted remains below the thyroid being bilateral.

Of the 22 subjects in whom the glands within the thymus or its involuted remains below the thyroid were bilateral, in 17 the right and left glands were on the same level. The glands within thymus III most frequently lay within its upper pole or formed its upper pole, but they sometimes lay beneath it. In 3 subjects a parathyroid was contained in the upper pole of a thymus III which ended short on that side at or just above the innominate vein. In the 3 subjects with a discrete thymus lobule III containing a parathyroid the lobule lay between the thyroid and the upper pole of the thymus. In only one of these three cases did the thymus III end short in the neck on that side, although that was the rule when a thymus lobule III was found in subsequent dissections.

In 3 subjects one parathyroid IV was contained in a discrete mass of thymus IV that lay behind the thyroid in the position of election, IV 1.

In 36 subjects connection between the thymic tissue and parathyroid was not seen with the naked eye but was found on microscopic examination. In 31 of these subjects thymic tissue was thus found to be connected with parathyroid III in 20, with parathyroid IV in 5, with both parathyroids III and IV in 1 and with unplaced parathyroids in 5. The thymic tissue often almost encapsulated the gland, from which it was usually separated by fibrous tissue. In several cases, however, the thymic tissue was completely fused with either parathyroid III or parathyroid IV. In two cases in this series and in two subsequent dissections Laidlaw's silver impregnation for reticulum fibres was used and showed very clearly such a direct continuity between the two tissues. When there was complete fusion lymphocytes from the cortex of the thymus sometimes extended into the perivascular connective tissue of the adjacent part of the parathyroid.

Further, fatty pads containing parathyroids from several subjects were examined microscopically, and in each of 5 subjects one such pad was found to contain thymic tissue, although none was recognisable with the naked eye.

Close association, sometimes fusion, between thymus and parathyroid was therefore seen with the naked eye or the microscope in 138 subjects in a series of 428, that is in 32 per cent. This comprises 96 subjects with glands in thymus III or its involuted remains, 3 with glands in thymus lobules III, 3 with glands in thymus IV, and 36 in whom microscopic examination showed association between parathyroids and portions of thymus III or IV, the thymic tissue being in fatty pads in 5.

Kurstemer (1898-99) and Getzowa (1907) found both upper and lower parathyroids in association with thymic lobules. Petersen (1903) and von Verebely (1907) also mentioned the macroscopic or microscopic association of parathyroid tissue and thymus. Erdheim (1904a) described parathyroids in the upper pole of thymus III and in thymus IV. In one the gland was fused with thymus IV and lymphocytes from the thymic cortex pervaded the neighbouring perivascular tissue of the gland. Gérard (1928) described invagination of the cortex of the thymus into the parathyroid with which it was fused.

*Phrenic nerve* Askanazy (1911) in a case of leprosy examined many nerves microscopically and found in a phrenic nerve a small mass of parathyroid tissue, in which he claimed to have found oxyphil cells. Four parathyroid glands in relation to the thyroid were also found.

### Size

The 1713 glands from the 428 subjects were among those employed for the determination of normal weights by Gilmour and Martin (1937), 316 were in cases regarded in their analysis as abnormal. The mean diameters of the remaining 1397 are set out in the following table.

Age stillborn to 1 day	34 glands	1.3 × 1.1 × 0.7 mm.
" 1 day to 3 months	14 "	1.5 × 1.1 × 0.7 "
" 3 months to 1 year	40 "	2.7 × 1.6 × 0.8 "
" 1 to 5 years	61 "	3.3 × 2.06 × 1.01 "
" 6 to 10 years	20 parathyroids IV	4.3 × 2.27 × 0.98 "
" " "	19 "	III 4.37 × 2.6 × 1.24 "
" 11 to 20 years	44 "	IV 5.25 × 3.0 × 1.28 "
" " "	42 "	III 5.31 × 3.26 × 1.43 "
" 21 to 30 years	46 "	IV 6.4 × 3.23 × 1.56 "
" " "	48 "	III 6.29 × 3.89 × 1.66 "
" 31 years and over—		
Males	300 "	IV 6.07 × 3.29 × 1.42 "
Females	200 "	IV 6.38 × 3.3 × 1.45 "
Males	301 "	III 6.50 × 3.55 × 1.6 "
Females	198 "	III 6.63 × 3.61 × 1.64 "

As would be expected these sizes show variations which agree with those in weight found by Gilmour and Martin to be associated with the age and sex of the subjects and the pouch of origin of the glands\*. As with weight there is no marked increase in size in the first three months of life. The maximum size is reached between 21 and 30 years. Female glands are larger than the corresponding male glands. Parathyroids III are larger than parathyroids IV in both males and females. In any gland the largest diameter was regarded as length, the next as width and the smallest as thickness. The two longest glands measured 22 and 18 mm., and were stretched over adenomata in the thyroid. A length of

\* In the paper upon which this is based it was impracticable to report the exact level at which the glands were found to be III or IV, and in the present tables the glands are grouped into these two categories.



to make the glands firmer. Glands excluded from the 'series because of hyperplasia were also firmer and more elastic. This change enabled hyperplasia to be recognised macroscopically in glands of normal size.

The glands usually sank in the 4 per cent saline formaldehyde used for fixation. In 64 subjects (15 per cent), however, one or more glands floated. It is obvious, therefore, that for differentiating parathyroids from adipose tissue the behaviour in water or dilute formaldehyde is a test of value only when the tissue sinks.

Benjamins stated that in goitre the glands may be stretched or otherwise deformed. He also described bilobed glands. Petersen stressed the presence of a hilum into which the vessels run.

### *Colour.*

The colour varied distinctively with age. In the new-born and up to the age of 3 months the glands were grey, glistening and semi-transparent, apparently due to oedema. About the end of this time the glands became greyish white, more opaque and not as a rule glistening. If marked wasting was present they sometimes remained for a considerable time of the size and appearance of those in the new-born. In the age-group 6-10 years, except in 3 of the younger subjects, a yellow tinge was apparent, sometimes a distinct creamy yellow. In age-group 11-20 years the adult appearance was developed. The colour was then predominantly yellow, but most glands had a brownish or pinkish brown element in the yellow. The glands thus contrasted with most of those seen on thyroids removed at operation, for these were almost pure yellow. Congestion made glands reddish brown or dark brown. Adiposity frequently affected most severely one end of a gland, which thus became more yellow than the other. In trimming glands care must be taken not to remove these adipose parts. Some glands which were lobulated and yellow appeared identical with adipose tissue, and one such gland floated in water. Adiposity is not, however, the essential factor in producing the yellow colour, because no fat was found on microscopic examination in some glands that were pure yellow. Conversely, owing to congestion extremely adipose glands were sometimes dark brown with no trace of yellow.

Yanase first described the characteristic transparency of infantile glands and the appearance later of opacity. Erdheim (1904b) described the enlargement and dark colour of congested glands.

### *Summary.*

A consecutive series of 428 dissections giving 1713 parathyroid glands established by microscopic examination is analysed, and





MULLER, L R	<i>Beitr path Anat</i> , 1896, xix 127
PEPERE, A	<i>Arch Ital Biol</i> , 1907-08, xlviii. 67
PETERSEN, H	<i>Arch path Anat</i> , 1903, clxxiv 413
ROSSLE, R	<i>Ibid</i> , 1932, cclxxxiii 41
SANDSTROM, I	<i>Upsala lakaref Forh</i> , 1879-80, xv 441, extract in Schmidt's <i>Jahrbucher</i> , 1880, clxxxvii 114
SCHAPER, A	<i>Arch mikr Anat</i> , 1895, xlv 239
SCHREIBER, L	<i>Ibid</i> , 1898, li 707
VON VEREBLLY, T	<i>Arch path Anat</i> , 1907, clxxxvii 80
WILSH, D A	<i>J Anat Physiol</i> , 1897-98, xxxii 292 and 380
YANASE, J	<i>Jahrb Kinderh</i> , 1908, lxxvii (Erg -Heft), 57
ZUCKERKANDL, E.	<i>Anat Hefte</i> , 1902, xix 61

### Appendix

#### *Method of dissection for discovering the parathyroids*

Owing to the close relation between thymus III and parathyroid III it is essential that the thymus or at any rate its upper poles should not be removed. It was therefore dissected from the pericardium as far upwards as the left innominate vein and was left hanging by its upper poles. The heart was examined and excised and then the remaining tissues of the neck and thorax were removed. The lungs were cut off through their hila, but the trachea and œsophagus were left unopened. The remainder of the specimen was examined from its posterior surface. As a rule no parathyroid glands were visible in the intact preparation. Then on the side to be examined an incision was made vertically through the fascia from the level of the bifurcation of the common carotid artery downwards over the posterior part of the thyroid about 1 cm. external to its posterior border, and was continued down on a deeper plane into the angle formed by the trachea and common carotid artery. The fascia over the thyroid was then retracted with the point of the scalpel towards the mid-line. Frequently parathyroid IV was thus revealed immediately. Otherwise a search was made for it with the middle of the posterior border of the thyroid as a starting point, the various positions in which the gland may be being explored. In searching for parathyroid III the lower pole of the thyroid was taken as the starting point. If it was not found thereabouts or if the upper pole of the thymus was not identified there, the thymus was traced up from below. The direction taken by the upper pole of the thymus often led to the gland even if the gland did not lie actually within it. Throughout the search for the glands all discrete pads of fat were carefully scrutinised, the variations in size, shape and colour which the glands may show being kept in mind. All minor exploratory incisions were made vertically with a sharp scalpel.

In identifying the glands the greatest difficulty was given by pigmented and somewhat wasted fat, but here the water test and the fact that parathyroids are rarely macroscopically lobular were helpful. Consistency was of the greatest help. In adults it should always prevent confusion with accessory thyroid tissue, but in infants accessory thyroids are so small that their consistency cannot be tested. In infants no difficulty was found in identifying parathyroids IV, which were often visible without incision into the fascia, but parathyroids III were often difficult to distinguish from accessory thyroids and lymph glands. It was sometimes impossible to



MULLER, L R	.	<i>Beitr. path Anat</i> , 1896, xix 127
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# AN EXPERIMENTAL STUDY OF PNEUMONIA FOLLOWING THE ASPIRATION OF OILY SUBSTANCES LIPOID CELL PNEUMONIA\*

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*University College Hospital Medical School, London*

(PLATES VI AND VII)

EVIDENCE is gradually accumulating that the aspiration of oily substances may lead to well defined pulmonary changes, generally known as lipoid cell pneumonia. Table I summarises the literature. It shows that not only the commonly used cod liver oil and liquid paraffin may be responsible but that less well known oils and fats may also be pathogenic. Table I also brings out the fact that provided conditions favourable to aspiration be present, lipoid cell pneumonia can occur in adults as well as in children.

Attention may be directed to certain of these cases. Fischer-Wasch (1912-33) for instance describes the typical change in a male with supposed catarrh who had had daily nasal installations of 0.1 per cent menthol in paraffin for 20 years. Bodner and Kallós's case (1933) suffered from catarrh and chronic pachydermia of the vocal cords. Every evening for 10 years he had poured 50 to 100 c.c. of liquid paraffin slowly down his nose, allowing it to trickle into his larynx. "Stimmänderung ölen zu können". The cough reflex was absent. Radiographic examination of his chest in 1927 suggested a basal bronchopneumonia. The shadows were unaltered five years later. In Laughlin's case (1926) the patient a male aged 74 years had paralysis of the palate, vocal cords and accessory laryngeal muscles. He could swallow when food was placed well back in the throat. He was given 1½ oz. of liquid paraffin three times daily for 18 weeks. Hauck (1936) reports the case of a male 47 years old who was treated for neutra bronchitis with intratracheal injections of warm liquid paraffin. The resulting illness persisted for 6 years and 8 months before proving fatal. Oil was repeatedly found in the sputum. Thomas and Jewett (1926) have reported oil pneumonia in an adult with cardiospasm and regurgitation following the aspiration of butter fat.

There is little difficulty in producing lipoid cell pneumonia experimentally. Cuvassio Pellissier (1920) injected olive oil intratracheally into rabbits and dogs and found the lung alveoli plugged with polymorphonuclear leucocytes and macrophages rich in oil droplets. Corper and Freed (1922) found that sunno oils, e.g. olive oil and liquid petrolatum, appear to be inert. These

\* Part of a thesis approved for the degree of Doctor of Medicine in the University of London.



remain in the lungs for a long time (months) producing at the most a simple proliferative reaction. Oils easily hydrolysed in the tissues or containing an irritant active principle produced pneumonia, consolidation or abscess formation. Iwajken produced lipoid cell pneumonia in rabbits by giving menthol in alkaline intratracheally over periods ranging from two days to a month, and also by nasal and pharyngeal instillation. Ikeda (1935), on the other hand considers that the purulent pneumonia associated with the aspiration of milk is due to its high bacterial content. A most extensive investigation has been carried out by Pinkerton (1929) who claimed that animal, mineral and vegetable oils produced different reactions although the members of each group gave a similar type of pneumonia.

Fatty material is of course found in the lungs in a number of conditions, e.g. fat embolism, lipemia associated with diabetes mellitus, the Hand-Schüller-Christian syndrome and Gaucher's disease. MacMillan and Weiss (1929) noted much fat in the pulmonary arteries in carbon tetrachloride poisoning. A few small globules of fat were present in the alveolar capillaries but not in the alveoli. The diagnosis is generally straightforward in such cases.

In 513 consecutive autopsies at University College Hospital eight cases of lipoid cell pneumonia have been found. Relevant details are summarised in table II. Frozen sections when available

TABLE II

*Cases of lipoid cell pneumonia encountered in a consecutive series of 513 autopsies*

Case	Age	Sex	Probable cause of aspiration	Nature of oily material	Lung changes
1	54 yrs	M	Cardiovascular and regurgitation	Liquid paraffin	Nodules 1.2 in in diameter, connective tissue invasion
2	Adult	M	?	?	Bilateral bronchopneumonia
3	54 yrs	M	Coronary	?	One large nodule 3 in in diameter, bilateral bronchopneumonia
4	59	M	Vomiting	Liquid paraffin	Advanced bronchopneumonia in left lower lobe
5	50	M	Conia	?	Bilateral bronchopneumonia in lower lobes practically confluent
6	57	M	?	?	Bronchopneumonia in both lower lobes and right middle lobe
7	2 wks	F	Convulsions	Breast milk	Edema and early bronchopneumonia of both lower lobes
8	2	M	Vomiting	Milk	Bronchopneumonia of both lower lobes

were stained for fats. In case 1 a lobe of the lung was extracted with chloroform and about 50 c of oily material obtained. This oil proved insoluble in acetone and alcohol, but soluble in ether and chloroform. It gave the same staining reactions as in the tissues except that with Nile blue sulphate a green fluorescence was obtained. The results correspond with the solubility, staining reactions and refraction of liquid paraffin and the higher members of the paraffin series.

TABLE I.  
*Oils known to have caused lepid cell pneumonia.*

Oil	Author	Date	Sex	Age	Conditions leading to aspiration
Cod liver oil	Pinkerton	1927	M	7 mths	Diarrhoea and vomiting
	Rabinovitch and Lederer	1934	M	4 yrs	Bulbar paralysis
	Goodwin	1934	M	11 mths	Gavage
	"	1934	F.	2 "	Vomiting
	"	1934	F.	11 "	"
	"	1934	M	2 yrs	Vomiting and convulsions
	"	1934	F	4 mths	Vomiting
	"	1934	F	7 "	Convulsions and regurgitation
	"	1934	M	5 "	Vomiting
	"	1935	F	18 "	Cleft palate and harelip
	"	1935	M	10 "	Vomiting and regurgitation
	"	1935	F	22 "	Cleft palate and harelip
Halibut liver oil	Laughlin	1925	M	3 yrs	Diabetes and coma
		1925	M	34 "	Multiple paralysis
		1927	M	6 "	Nasal instillation
	Pinkerton	1936	M.	47 "	Intratracheal injection
	Houck	1932-33	M	Adult	Nasal instillation
	Fischer-Wasels	1933	M	41 yrs	"
	Bodmer and Kallós	1935	M	3 mths	"
	Ikedá	1935	M	10 "	Regurgitation
	"	1937	M	58 yrs	Cardiospasm and regurgitation
	This paper				
Cod liver oil and liquid paraffin	Goodwin	1934	M	4 mths	Vomiting
	"	1934	F	4 "	"
	Ikedá	1935	F.	10 "	Nasal instillation
	"	1935	F	3 "	Regurgitation
Albino	Laughlin	1925	F.	2½ yrs.	"
	"	1925	M.	Infant	Nasal instillation
Butter fat (cream)	Thomas and Jowett	1926	M.	Adult	"
					Cardiospasm and regurgitation

up by mononuclear cells (macrophages) There were some large free masses of oil in the peribronchial lymphatic tissue These stained pink with Nile blue sulphate and were therefore not liquid paraffin These masses were found in about 75 per cent of the rats examined they are found in normal animals (Seemann, 1929-30) Paraffin sections showed small areas of consolidation in which the alveoli were filled partly with exudate, partly with macrophages distended with droplets of oil of greatly varying size Some of these cells contained only minute droplets which gave them a foamy appearance The alveolar capillaries were congested The peribronchial lymphatics were distended and contained macrophages with ingested oil (fig 1) There were some polymorphonuclear cells in a few of the bronchioles

*27 days* Lungs pale and firm Surface irregular with small patches of compensatory emphysema A few greyish nodules projected from the lung surface near the bases Frozen sections showed that the greater part of the oil had been phagocyted The free masses in the alveoli were fewer in number The distribution of the oil was still patchy Paraffin sections showed areas of consolidation in which the alveoli were stuffed with macrophages showing certain definite characteristics, viz the cells were enlarged and contained several large globules of oil which displaced and distorted the nucleus so that it might be eccentric and crescent shaped (fig 2) Figure 3 shows similar cells in case 1 of our autopsy series The alveolar congestion was much less and no exudate could be found Some oil, both free and in macrophages, was found in the interstitial tissue of the alveolar walls, which in the consolidated areas showed some hyperplasia and increased formation of collagen No giant cells were seen The peribronchial lymphatics were not distended

*70 days* Lungs small with numerous small patches of collapse No solid nodules Pleural cavities normal In frozen sections the irregular distribution of the oil in small patches was still a noticeable feature By far the greater part of the oil however was phagocyted Paraffin sections showed slight consolidation and emphasised the hyperplasia of the alveolar walls Well formed giant cells were present in the pneumonic areas, especially near the peribronchial lymphatic tissue Some were small and contained only two or three nuclei, others were large syncytial masses with up to fifteen nuclei (fig 4)

A rabbit was killed 27 days after 5 c.c. of liquid paraffin had been administered intratracheally Macroscopically the lungs showed changes of bronchopneumonia Sections showed an irregular consolidation of the alveoli with oil-laden macrophages There was no evidence of purulent bronchopneumonia The alveolar walls showed hyperplasia and giant-cell formation was



The experimental work described in this paper arose out of our interest in lipoid cell pneumonia, stimulated by its high incidence in our autopsy material. The object has been to reproduce in animals as far as possible lesions seen in the human subject. Animal, fish, mineral and vegetable oils were administered intratracheally and the resulting lesions studied at different stages of development. Special attention has been directed to the possibility that differences in constitution of the oil might determine variations in reaction.

### Methods

The following substances have been studied

- 1 *Vegetable oils* Olive oil, linseed oil, castor oil, poppy seed oil, arachis oil
- 2 *Fish oil* Cod liver oil
- 3 *Animal oils* Butter fat, milk
- 4 *Mineral oils* Liquid paraffin, diesel engine gas oil, transformer oil
- 5 *Esters of saturated fatty acids*
  - a *Short chain* Tributyrin
  - b *Long chain* Tripalmitin
- 6 *Esters of unsaturated fatty acids*
  - a *Short chain* Triolein
  - b *Long chain* Methyl ester of lauric acid, ethyl ester of linoleic acid
- 7 *Sterols* Cholesterol
- 8 *Phosphatides* Lecithin

Sixty-five Wistar albino rats, fully grown (200-300 g weight), both male and female, were lightly anaesthetised with ether and an intratracheal injection of the oil given with a syringe and blunted needle. When the fat was solid an emulsion was made in water from an alcoholic solution. Control rats were anaesthetised but no oil was injected. 0.1 to 0.2 c.c. was given on 7 consecutive days and the animals killed 7, 27 and 70 days afterwards. Seven rabbits, average weight 2 kg., were given one injection of 5 c.c. of oil through an intratracheal catheter without anaesthetic.

The lungs were fixed in 10 per cent formal saline. Frozen sections were stained with Scharlach R and haematoxylin or methylene blue, paraffin sections with Ehrlich's acid haematoxylin and eosin and by van Gieson's method. All the animals were quite healthy during the course of the experiments and presented no naturally occurring disease at death. The bronchiectatic lesions occurring spontaneously in underfed rats (Passey, Leese, and Knox, 1936) were never found, although mild degrees of bronchial dilatation following blocking of the smaller bronchi by oil globules were noticed in some animals and are described. The rats were fed on a diet adequate in vitamins A, C and D and grew normally throughout the experiments.

### Results

**Liquid paraffin. 7 days** Surface of lungs reddened, especially the lower lobes. Small shotty nodules in lower half of left lobe. Tiny solid greyish translucent patches on posterior surface of left lobe. Frozen sections showed a patchy distribution of the oil, some of it lying free in the alveoli and some in finer droplets taken

LIPID CELL PNEUMONIA

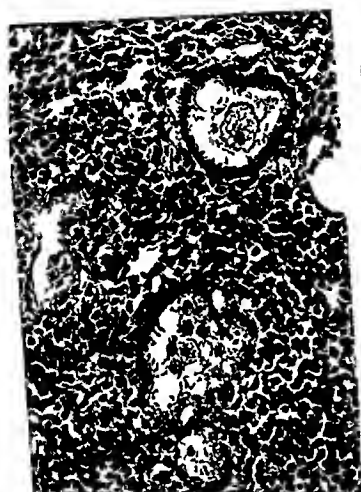


FIG 1—Liquid paraffin dilated peribronchial lymphatics containing oil laden macrophages Rat X235



FIG 2—Liquid paraffin characteristic distortion of macrophages by the ingested oil droplets Rat X235



FIG 3.—Case 1 Liquid paraffin cells filled with coarse droplets of oil X235

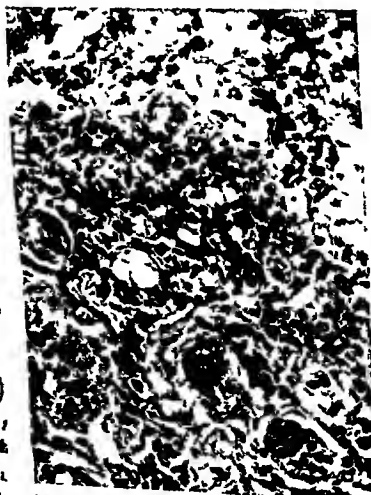


FIG 4—Liquid paraffin formation of multinucleated giant cells Rat X235

noted in the consolidated areas. Sections were cut of the hilar lymph glands but no fatty material was found

*Summary* Intratracheal administration of liquid paraffin to rats and rabbits leads to the formation of well defined nodules in the lungs. These are at first composed of macrophages but later on giant cells and connective tissue appear. Phagocytosis of oil globules is marked and occurs in a characteristic manner.

*Diesoline gas oil* Some of the animals died 12 hours after injection. They showed gross congestion and oedema of the lungs. The oil lay in the alveoli in large and small free masses. Paraffin sections showed acute congestion and hæmorrhage into the alveoli with large numbers of polymorphonuclear leucocytes and macrophages. Oil globules were found in the cartilage cells of the bronchi of a rabbit.

*7 days* Lungs normal in colour and size with greyish nodules in the lower lobes. Frozen sections showed large patches of consolidated lung tissue containing oil, some lying free and some engulfed in phagocytes. Paraffin sections showed macrophages indistinguishable from those found in lesions produced by liquid paraffin. Collections of polymorphonuclear cells were seen in some alveoli. Droplets of oil were present in the interstitial tissue.

*32 days* Lungs appeared normal except for several grey nodules projecting from the surface of all lobes. Frozen sections showed only a little free oil. Most of it was in a finely divided state within macrophages. Paraffin sections showed a picture similar to that produced by liquid paraffin.

*Summary.* In large amounts diesoline is intensely irritant to the lungs. In animals which survive, the pulmonary reaction closely resembles that with liquid paraffin.

*Transformer oil. 7 days.* Lungs showed a few small greyish patches of consolidation near the surface of the right lower lobe, otherwise they were normal. In frozen sections an irregular distribution of the oil, which was mostly lying free in the alveoli, was observed. There was very little cellular reaction.

*30 days.* Lungs apparently normal except for some small areas of collapse. Very little oil was found in the frozen sections and there was no evidence of any reaction on the part of the lung tissue.

*70 days* Lungs slightly emphysematous, some golden brown speckling beneath the pleura of the lower lobes. On section there appeared to be small patches of consolidation. In frozen sections the oil was irregularly distributed and mainly intracellular. The globules were both large and small and the appearance of the macrophages varied from a granular type to one showing large globules as in liquid paraffin lesions. The macrophages were chiefly in the alveolar walls. No giant cell reaction was seen.

*Summary* Transformer oil produces very little reaction in the lungs of experimental animals even after the intratracheal introduction of relatively large amounts

*Cod liver oil 7 days* Lungs partially collapsed at both bases with some small yellowish nodules at the base of the right lower lobe. In frozen sections oil was present as free masses in the alveoli. Very few macrophages were seen. Paraffin sections showed areas of consolidation chiefly around the bronchi and bronchioles. In the alveoli was a loose shreddy exudate surrounding oil spaces and entangling small macrophages. Those cells which contained oil had a granular appearance due to the extremely finely divided state of the oil and presented quite a different appearance from those seen in the liquid paraffin lesions. The peribronchial lymphatics were not dilated and there was no purulent inflammation of the lung tissue.

*27 days* Lungs pale, small and firm. Scattered over the posterior surface were many subpleural greyish nodules. Frozen sections showed the oil to be finely emulsified and phagocytosed. A few larger masses of free oil were seen. In the paraffin sections there was irregular patchy consolidation, the alveoli being filled with macrophages. These cells had a granular appearance due to the very fine state of division of the oil (fig 5), and resembled the cells seen in case 8 (fig 6). Some hyperplasia of the alveolar walls and congestion of the alveolar capillaries was observed. There was no giant cell reaction.

*70 days* Lungs shrunken, with small raised patches of compensatory emphysema. Some yellowish discolouration of the surface of the lower lobes with many pin-point greyish nodules. Frozen sections showed some loose masses of oil in the alveoli but most of it had been phagocytosed. Paraffin sections brought out in addition an increase in the number of collagen fibrils in the alveolar walls and small giant cells were seen.

*Old cod liver oil* Rats were given one injection of 1 c.c. of cod liver oil about two years old which had developed a rancid biting odour due to the presence of a peroxide. After 28 days the pulmonary lesions were about the same in nature and extent as those produced by fresh cod liver oil. Bronchiectasis was however well marked and many of the macrophages appeared to be fusing into small syncytial masses with several nuclei.

*Summary* Cod liver oil produced a well marked nodular reaction consisting of a macrophage accumulation around the introduced oil masses. Phagocytosis proceeded actively in a characteristic manner different from that seen with liquid paraffin. Fibrosis occurred in the later stages but was not marked.

Olive oil produced a nodular reaction, well defined in seven days but noticeably diminished in 70 days. The nodules were

composed of macrophages, some filled with large droplets of oil, others stuffed with tiny oil granules. The cellular reaction was not very marked, nor was there evidence of giant-cell formation or fibrosis.

**Neohydriol** (poppy seed oil containing 40 per cent iodine) called forth very little reaction. The macrophages took on a finely granular appearance after phagocytosis of the oil. Apparently most of the neohydriol was got rid of by other means.

**Castor oil** produced a very slight reaction, most of the oil being removed by methods other than phagocytosis. Macrophages showed a finely granular appearance similar to that seen with cod liver oil.

**Arachis oil** produced a well marked pulmonary reaction, macrophages invading the oil masses and taking on a granular appearance through fine emulsification of the oil. Capillary congestion was more marked and a fibrinous exudate appeared in the alveoli.

**Linseed oil** was associated with a fairly intense congestion and exudation in the lungs during the early stages, but macrophages were seldom numerous. Phagocytosis was not marked, the phagocytes had a finely granular appearance. In the later stages fibrosis was present though not marked. Giant cells were not seen.

**Cow's milk** produced no lasting change, lungs examined 7 and 56 days after intratracheal administration showing no definite lesion. Both boiled and untreated milk were used. The bacteria present were apparently not pathogenic to rats. The fat content of the milk was very low (less than 5 per cent).

**Butter** Injected at body temperature, butter produced a slight macrophage response after 7 days and small greyish nodules were seen after 56 days. The fat was ingested by phagocytes which became filled with fine granules of oil. There was considerable increase in fibrous tissue in some of the lungs and usually marked proliferation of the peribronchial lymphatic tissue. Whilst free fat was present in the alveoli the lymphatics appeared widely dilated.

**Tributyrin.** Rats were given one injection of 1 c.c. intratracheally. One rat died five minutes after injection with acute œdema of the lungs.

**28 days.** Lower lobes of lungs studded with greyish nodules many of which were around the bronchi. Frozen sections showed areas of bronchiectasis with numerous oil-laden macrophages in the peribronchial lymphatic tissue and also in the exudate in the bronchial lumina. Paraffin sections showed advanced peribronchial fibrosis but no alteration in the type of bronchial epithelium. Many macrophages still contained oil in a very fine state of division. Some had fused to form small syncytial masses. In this case no oil was found except in the

## LIPOID CELL PNEUMONIA

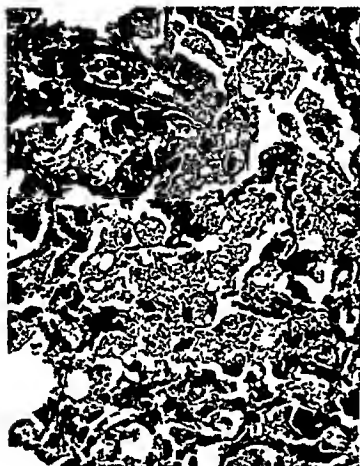


FIG. 5—Cod liver oil granular appearance of the macrophages produced by fine emulsification of the oil Rat  $\times 235$



FIG. 6—Case 8 Milk cells containing finely emulsified oil similar to those produced experimentally by cod liver oil  $\times 235$

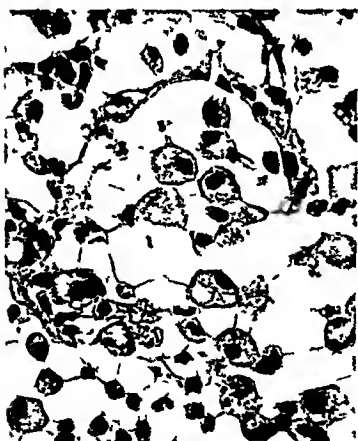


FIG. 7—Lecithin alveolar exudate showing granular macrophages and polymorphonuclear leucocytes Rat  $\times 375$



FIG. 8—Lecithin concentration of the fatty material around the greatly hypertrophied peribronchial lymphatic tissue Rat Sudan III  $\times 45$

composed of macrophages, some filled with large droplets of oil, others stuffed with tiny oil granules. The cellular reaction was not very marked, nor was there evidence of giant-cell formation or fibrosis.

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bronchiectatic lesions and it seems reasonable to suppose that, with the large injection given, blockage of a bronchus had occurred. No bronchiectatic lesions were seen except in conjunction with masses of fat. There was no evidence of rat pneumonia.

**Tripalmitin** A rabbit was given 5 c.c. intratracheally.

**28 days** Lungs appeared normal. Frozen sections showed a few scattered oil droplets, very few of which were phagocytosed. There was practically no emulsification. The cartilage cells around one of the bronchi contained many globules of fat. In paraffin sections no oil reaction was found. There were many areas of collapse with some fibrosis of the alveolar walls.

**Summary** Tributyrin is toxic to some animals and they may die from acute oedema of the lungs. The fat is finely emulsified and ingested by macrophages. Tripalmitin produces very little reaction in the lungs so far as can be seen from the limited material.

**Triolein** Rats were injected with a single dose of triolein of about 1 c.c.

**30 days** Lungs appeared normal. Frozen sections showed a few large globules of oil which had escaped phagocytosis. Paraffin sections showed only a few small areas of collapse.

**59 days** Lungs normal except for some tiny nodules along the edge of the right middle lobe. Frozen sections showed only minute amounts of oil, partly phagocytosed, partly in free masses.

**Ethyl ester of linoleic acid** Rats were given one injection of 1 c.c. One rat died after 18 hours with acute congestion and nodular consolidation of the lung. In frozen sections much oil was scattered evenly throughout. It was chiefly in shreddy masses, while some had been broken up into small globules but not phagocytosed. Paraffin sections showed an acute oedema with many small macrophages in the alveoli. Two other rats were killed 45 days after injection but no oil was found in the lungs.

**Methyl ester of linoleic acid** Rats were given one injection of 1 c.c.

**10 days** The lungs showed some solid white patches raised 1 mm. above the surface in the left lobe. Frozen sections showed that the exudate in the consolidated areas contained polymorphonuclear cells and macrophages stuffed with fine droplets of oil. In paraffin sections were areas of pneumonia in which the alveoli were filled with structureless exudate and many polymorphonuclear cells and macrophages of the granular type. Oil was found only in these consolidated areas. Elsewhere there were patches of collapse and emphysema.

**45 days** Lungs normal. No oil was found in frozen sections and paraffin sections showed normal tissue.

**Summary** The esters of unsaturated fatty acids are rapidly removed from the lungs. Triolein is only slightly phagocytosed.





arise in three ways (1) through the administration of oil to young infants in whom the swallowing reflex may not be fully developed, (2) in patients who have laryngeal paralysis or some pathological condition which may lead to vomiting, regurgitation or coma, in this group the pulmonary lesion may arise from aspiration of fat containing stomach contents, and (3), surprisingly frequently by nasal instillation of oil for some catarrhal condition of the nose and throat, it would seem that nasal administration of oily substances, especially to individuals with altered laryngeal reflexes, should not be lightly undertaken.

The pathological picture when fully developed is typical. The presence of a fairly severe bronchopneumonia in which small and large, prominent, greyish or white firm areas project from the lung substance is suggestive of lipoid cell pneumonia. The diagnosis from tuberculosis or malignant disease of the lung presents little difficulty, for the distribution and character of the lesions are different. But the fully formed picture is not a constant finding, and in all likelihood depends on a considerable amount of oil having been aspirated. Thus in our 8 cases 2 only, both adults, showed easily recognisable white nodules. In one the nodules were half-an-inch to 2 inches in diameter, finely trabeculated from invasion of connective tissue, and showed tiny cystic spaces some of which were filled with soft white material. In the second case a large, firm, white area with central softening was found at the base of one lung. In the other 6 cases no such nodules were found and the diagnosis was made from routine microscopical preparations some time after the autopsies had been performed. It is possible that, with the experience obtained from a study of the two cases with characteristic nodules, some of these earlier cases might have proved no less typical. In the majority, the usual finding was severe bronchial pneumonia with much exudate in the bronchi. Pulmonary oedema was absent except in a two weeks' old child. Another case in a sixteen days' old infant showed no oedema of the lungs. Enlargement of the hilar and tracheo-bronchial glands, though found in two cases, was more frequently absent.

The ultimate diagnosis depends on the microscopic examination of frozen and paraffin sections. Fat staining settles the nature of the free or phagocytised masses of foreign material which can usually be detected in the alveoli of the lungs. Fat staining may also give a clue to the type of oily substance aspirated. Pinkerton (1927) gives a very useful table of the staining reactions, solubilities and refraction of a number of the common oily substances which may reach the lungs.

In addition to these tests valuable information is obtained from a study of the phagocytes (macrophages), especially the manner in which they have ingested oil globules. My experiments

The long chain esters are finely emulsified and are partly removed by phagocytosis

**Lecithin** Rats were given a single dose of 1 c c intratracheally in watery solution

**28 days** The left lobe of the lung showed several patches of consolidation and some areas of bronchiectasis. In frozen sections the oil, finely emulsified, was distributed around bronchi and was phagocytosed. In the dilated bronchi were polymorphonuclear cells and oil-containing macrophages embedded in a mucinous exudate. The bronchial epithelium was normal. Paraffin sections showed areas of consolidation in which the exudate contained many macrophages of granular type and some polymorphonuclear leucocytes (fig 7). There was no hyperplasia of the alveolar walls. The peribronchial lymphatic tissue was greatly increased in the bronchiectatic areas.

**59 days** There was some brownish speckling of the lung surface and the lungs felt firm. Frozen sections showed marked hyperplasia of the peribronchial lymphatic tissue but no bronchiectasis in the material examined. The bronchial mucosa was hypertrophied and folded into the lumen. The fatty material was finely emulsified and phagocytosed by macrophages collected in groups around the bronchi (fig 8).

**Summary** Lecithin produces marked peribronchial cellular response and hyperplasia of the lymphatic tissue.

**Cholesterol** Rats were given a single injection of 1 c c of an emulsion of cholesterol.

**10 days** Lungs appeared normal. Frozen sections showed few but relatively large masses of cholesterol, there was no evidence of phagocytosis.

**30 days** Lungs appeared normal. In frozen sections small globules of doubly refracting fat were present in the extreme periphery of the lung. No phagocytosis was observed.

**40 days** Lungs normal. Sections showed some cholesterol at the periphery of the lung, some of it intracellular. The remainder of the lung substance was normal.

**Summary** Cholesterol appears to produce very little reaction in the lung. Most of it disappears rapidly from the pulmonary tissue and that which remains calls forth only a slight cellular reaction.

### *Discussion.*

Oil aspiration pneumonia occurs not only in infants but in adults of all ages. Consideration of the literature, together with our own cases, shows that it should be suspected in bedridden subjects suffering from paralysing diseases or disease associated with vomiting who develop pneumonia. Especially suggestive is a history of the administration of oily substances. Aspiration may

arise in three ways (1) through the administration of oil to young infants in whom the swallowing reflex may not be fully developed, (2) in patients who have laryngeal paralysis or some pathological condition which may lead to vomiting, regurgitation or coma, in this group the pulmonary lesion may arise from aspiration of fat-containing stomach contents, and (3), surprisingly frequently by nasal instillation of oil for some catarrhal condition of the nose and throat, it would seem that nasal administration of oily substances, especially to individuals with altered laryngeal reflexes, should not be lightly undertaken.

The pathological picture when fully developed is typical. The presence of a fairly severe bronchiopneumonia in which small and large, prominent, greyish or white firm areas project from the lung substance is suggestive of lipoid cell pneumonia. The diagnosis from tuberculosis or malignant disease of the lung presents little difficulty, for the distribution and character of the lesions are different. But the fully formed picture is not a constant finding, and in all likelihood depends on a considerable amount of oil having been aspirated. Thus in our 8 cases 2 only, both adults, showed easily recognisable white nodules. In one the nodules were half-an-inch to 2 inches in diameter, finely trabeculated from invasion of connective tissue, and showed tiny cystic spaces some of which were filled with soft white material. In the second case a large, firm, white area with central softening was found at the base of one lung. In the other 6 cases no such nodules were found and the diagnosis was made from routine microscopical preparations some time after the autopsies had been performed. It is possible that, with the experience obtained from a study of the two cases with characteristic nodules, some of these earlier cases might have proved no less typical. In the majority, the usual finding was severe bronchial pneumonia with much exudate in the bronchi. Pulmonary oedema was absent except in a two weeks' old child. Another case in a sixteen days' old infant showed no oedema of the lungs. Enlargement of the hilar and tracheo-bronchial glands, though found in two cases, was more frequently absent.

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In addition to these tests valuable information is obtained from a study of the phagocytes (macrophages), especially the manner in which they have ingested oil globules. My experiments

lead me to believe that cod liver oil is phagocytosed differently from liquid paraffin (*cf* figs 5 and 2), and I have found some evidence that these differences apply also to human material. In long-standing cases foreign body giant cells appear and may contain oil droplets. A slight connective tissue proliferation is sometimes seen but it is to be emphasised that this is not a prominent feature. The changes are essentially alveolar and bronchial in distribution.

There is close agreement between the changes in naturally occurring cases of oil aspiration pneumonia and those produced experimentally in animals. In experimental oil pneumonia, greyish or white areas and small nodules associated with bronchopneumonia and much exudate in the larger air passages are characteristic. Hilar gland changes too are slight. The fats can generally be demonstrated in the alveoli. Some however are quickly removed by the normal clearing mechanism of the lung—ciliary movement, bronchial peristalsis and perhaps absorption. Macrophages are numerous in the air spaces and are filled with oil particles. Giant cells may be formed after some time and a slight fibrous tissue reaction has been observed. The analogy between the experimental and human oil pneumonia is thus complete. The experimental study is of value in showing the similarity of reaction produced by various oils and their constituent compounds. At the same time it clearly suggests that individual variations are determined mainly by the rate of removal of the oil from the air passages. With cod liver oil and liquid paraffin consolidation may last for some months and eventually a proliferative change with formation of giant cells and fibrous tissue sets in. Any oil which remains in the lungs for a long time may produce such a reaction. Poppy seed oil (lipidol, neohydriol) is especially interesting. This oil is hardly phagocytosed at all, and at any rate in human beings is removed almost completely by expectoration, hence its suitability for radiographic work. Wright (1935), however, reports a case of carcinoma of the bronchus in which the tumour proved an effective barrier to the natural removal of the lipidol by expectoration and at autopsy an oil reaction was found. Also in experimental animals injected with neohydriol tiny areas of reaction were found after 70 days.

The vegetable oils as a group produce less reaction than the mineral, fish and animal oils, and are rapidly removed from the lungs. Arachis oil and olive oil lead to more marked lesions than other members of the group.

In considering the differences in phagocytosis of oil droplets, it would seem that the degree of emulsification is important. Liquid paraffin is coarsely emulsified and the distortion of the cells by relatively large droplets of oil is characteristic. Cod liver oil is finely emulsified and the ingested oil produces a granular or fluffy type of macrophage which is again distinctive. Roughly the oils

may be divided into three groups according to their degree of unsaturation Table III has been compiled to compare the degree

TABLE III

*Comparison of degree of emulsification with degree of unsaturation*

Degree of unsaturation	Oil	Degree of emulsification
Mainly saturated	Liquid paraffin Transformer oil Tributyrin Tripalmitin	Coarse . Fine Coarse
Mainly unsaturated	Cod liver oil Castor oil Arachis oil Linseed oil Poppy seed oil Triolein	Fine " . . . Coarse
Equal amounts of saturated and unsaturated groupings	Olive oil Butter fat Dicosine Lecithin	Coarse and fine Mostly fine Coarse and fine Fine

of emulsification with the degree of unsaturation It will be seen that the more unsaturated the oily substance, the more completely is it phagocytized within the lung There are two exceptions to this, tributyrin and triolein Tributyrin, being a glyceride of a saturated fatty acid, should be coarsely emulsified and triolein should be finely emulsified as it is unsaturated Obviously other factors are concerned in phagocytosis, but it does appear that there may be some relationship between emulsification, phagocytosis and the degree of unsaturation

In rats given a single large dose of oil there is a tendency for bronchiectatic lesions to develop That these lesions are due to bronchial changes produced by oil and not a naturally occurring disease seems probable because (1) bronchiectasis was never found in rats given a series of small doses of oil, (2) oil was found in the bronchi and surrounding lung tissue in all the bronchiectatic areas observed, (3) control animals were free from bronchiectasis and lung lesions No parallel can be drawn here between human and experimental observations, for so far as I know bronchiectasis has not been found in association with lipid cell pneumonia in man

### *Summary*

Lipoid cell pneumonia can be produced experimentally by the intratracheal administration of oil Relatively large quantities given in a series of small doses are required to produce a typical reaction, single large doses tend to produce bronchiectasis The reaction is similar with all oils and is essentially a macrophage

response with phagocytosis of oil droplets With very slow removal of the oil, a foreign body reaction may develop producing giant cells and fibrosis Vegetable oils are most easily removed from the lungs; liquid paraffin and cod liver oil produce the most severe reactions Some oils are very toxic, tributyrin for instance causing acute oedema of the lungs and diesel gas oil acute hæmorrhage into the alveoli Certain fractions of the oils produce the same reaction as the oil itself Lecithin is especially potent, cholesterol gives very little reaction The more unsaturated the oily substance the more finely is it emulsified within the lung and the more marked is the resulting phagocytosis

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# INFECTION OF MICE WITH *BRUCELLA ABORTUS* OF BOVINE ORIGIN \*

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HITHERTO guinea-pigs have been the most commonly used laboratory animals for experimental purposes and for the isolation of *Br abortus* from naturally infected material. In several recent publications, however, it has been suggested that it might be advantageous to use mice for such work, particularly in experiments where large numbers of animals are required.

That mice are susceptible to infection with *Br abortus* (bovine type) has been shown by Holtz (1911), Fabry (1912, 1913) and Hagan (1922). More recently, Ber (1933) has found that the inoculation of large numbers of organisms— $800 \times 10^6$ —gives rise to a septicaemic condition in mice which appears within 24 hours, and that it is possible to recover organisms from the blood for periods of 6 months or more. He also states that as few as 8 or 9 bacilli may be sufficient to cause infection. Further, in 6 cases where the inoculum consisted of 0.5 c.c. of the stomach contents of aborted calves, he was able to recover *Br abortus* from the spleens of inoculated mice after one month. Kildman and Olson (1935) inoculated mice with strains of bovine and porcine origin. Practically all survived the period of the experiment up to 70 days and *Br abortus* was recovered from the spleens of 28 out of 34 of the mice. Agglutinins to a titre regarded as significant were present in the sera of nearly all the animals whose blood was tested. Singer and Shaw (1935) report that inoculation with large doses, viz. 300 million *Br abortus* (porcine type), was fatal to white mice within 4 days. Similarly Singer Brooks (1937), working with *Br melitensis* and *Br abortus* (bovine and porcine types), found that injection of doses as high as 600 million organisms of the bovine type caused death of the mice within 4 days. It appears from this work that *Br melitensis* and *Br abortus* (porcine type) are more pathogenic for mice than is *Br abortus* (bovine type). Injection of *Br abortus* (bovine type) in lower dosage, viz. 100,000 and 1000 organisms, was not fatal to the mice and attempts at recovery of the organisms from injected animals were in most cases unsuccessful.

The present investigation was undertaken with a view to ascertaining the suitability of mice for experimental and diagnostic procedures in connection with *Br abortus* infection in cattle. Mice and guinea-pigs were inoculated with cultures of *Br abortus* of bovine origin or with naturally infected milk in order to compare their relative susceptibilities to infection.

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### Technique

Two cultures of *Br. abortus* (B 54 and Ast 370) isolated from aborted calves were used. The cultures for inoculation were grown on 5 per cent potato extract agar slopes in an atmosphere containing 10 per cent of CO<sub>2</sub>. The growth after 3 days was washed off in sterile Ringer's solution and standardised to tube 4 of Brown's opacity standards, which is equivalent to 780 million *Bact coli* per c.c. The inocula were prepared by diluting this suspension 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-10</sup>. Groups of fully grown mice and guinea-pigs were then injected with this material, each animal receiving 1 c.c. of the appropriate dilution. Mice were inoculated intraperitoneally or subcutaneously and guinea-pigs intramuscularly in the thigh. The number of organisms inoculated in each case was estimated by mixing 1 c.c. of the dilutions 1/10<sup>7</sup> to 1/10<sup>10</sup> with potato extract agar in Petri dishes. Counts were made after 3 days' incubation and the number of organisms in each dilution calculated.

The naturally infected milk was obtained from cows whose blood serum was showing a high agglutination titre for *Br. abortus*. Twenty c.c. of milk from each quarter were mixed and centrifuged for 15 minutes on a high-speed centrifuge. The cream was then collected with a wide-mouthed pipette and added to the deposit at the bottom of the centrifuge tube after decanting the supernatant milk. A few c.c. of this milk were utilised to facilitate the mixing of the cream and deposit. Four mice and 2 guinea-pigs were each inoculated with 1 c.c. of mixed cream and deposit from each of the cows.

In the experiments with cultures of *Br. abortus* the animals were killed approximately 60 days after inoculation. Blood was collected from the chest or axilla and was later submitted to an agglutination test against a *Br. abortus* suspension standardised by means of a dried *abortus* serum (Stableforth, 1936). The abdomen was opened and the whole of the spleen in mice, or part of the spleen in guinea-pigs, removed to a test-tube containing sterile broth and sand. The spleen was ground up by means of a small glass pestle and a loopful of the supernatant fluid then transferred to 2 slopes and 2 plates of potato extract agar. These were examined after 4-5 days' incubation and a number of colonies picked from each and sown on a further series of slopes. The resulting growths were washed off and submitted to the agglutination test with a known positive bovine serum. Diagnosis of infection was based on the recovery of *Br. abortus* from the spleen.

With naturally infected milk the post-mortem technique was similar, except that the mice and guinea-pigs were killed after the shorter interval of 30 days.

### Results.

**Expts I and II.** In these two experiments 62 mice in all were injected with doses ranging from  $810 \times 10^6$  to  $572 \times 10^2$  organisms from a culture of B 54. The results are combined in table I.

*Br. abortus* was recovered from the spleens of 33 out of the 51 mice which survived until the end of the experiments. Eleven mice died during the course of the experiments and from 7 of these *Br. abortus* was recovered. In two of the mice which died, all cultures from the spleen were grossly contaminated and in the result these two animals have been disregarded. Thus, of 60 mice in which a diagnosis was possible *Br. abortus* was recovered from the spleens of 40 animals.

TABLE I

*Susceptibility of mice to varying numbers of Br abortus (expts I and II)*

Number of organisms inoculated	Number of mice				
	In group	surviving	dying	infected	not infected
$810 \times 10^4$	10	14	5	14	5
$572 \times 10^4$	6*	3	3	4	1
$810 \times 10^3$	13	13	0	5	8
$572 \times 10^3$	0*	4	2	5	0
$572 \times 10^2$	0	0	0	6	0
$572 \times 10^1$	0	5	1	4	2
$572 \times 10^0$	0	0	0	2	4

\* All cultures from the spleen of one mouse in each of these groups were contaminated

Expt III Culture material prepared from strain B 54 of *Br abortus* was again used in this experiment. Groups of mice and guinea-pigs were injected with doses of organisms varying from  $280 \times 10^4$  to 28 organisms. The results are set out in table II.

TABLE II

*Susceptibility of mice and guinea pigs to Br abortus (bovine type)*

Number of organism inoculated	Strain used	Number of mice			Number of guinea pigs		
		in group	infected	not infected	in group	infected	not infected
<b>Expt III</b>							
280 × 10 <sup>4</sup>	B 54	10	10	0	4	4	0
280 × 10 <sup>3</sup>	B 54	13	13	0	5	5	0
280 × 10 <sup>2</sup>	B 54	15	14	1	5	5	0
280 × 10 <sup>1</sup>	B 54	14	13	1	4	4	0
280 × 10 <sup>0</sup>	B 54	11	15	10	5	5	0
280 × 10 <sup>-1</sup>	B 54	11	0	11	5	5	0
280	B 54	0	0	9	5	3	2
28	B 54	13	0	13	4	3	1
0*	B 54	14	0	14	5	0	5
<b>Expt IV</b>							
740 × 10 <sup>4</sup>	Ast 370	13	11	2	3	3	0
740 × 10 <sup>3</sup>	Ast 370	14	13	1	5	5	0
740 × 10 <sup>2</sup>	Ast 370	10	7	3	4	4	0

\* The inoculum contained no organisms as judged by the culture test

*Br abortus* was recovered from the spleens of 65 mice, or 56 per cent, of the 116 inoculated with bacteria and from 34 of the 37 guinea-pigs. The lowest dose with which mice were infected was  $280 \times 10^2$  organisms, whilst infection was found in guinea-pigs with a dose as low as 28 organisms. A further 14 mice and 5 guinea-pigs received an inoculum in which no organisms could be detected by plate cultures. In none of these animals was *Br abortus* recovered *post mortem*.

Expt. IV. Cultures were prepared from another strain of *Br. abortus* (Ast 370) Thirty-seven mice and 12 guinea-pigs received doses ranging from  $740 \times 10^6$  to  $740 \times 10^4$  organisms. Unfortunately, lower doses could not be used. *Br. abortus* was recovered from the spleens of 31 mice and from all the guinea-pigs (table II)

Expt. V. In this experiment the inoculation material consisted of the cream and the deposit from samples of milk from 10 cows infected with *Br. abortus* Four mice and 2 guinea-pigs were each injected with 1 c.c. of the material from each sample As a result of the guinea-pig inoculation the presence of organisms in the milk was diagnosed in all the samples (table III) Two guinea-pigs

TABLE III.

*Inoculation of mice and guinea-pigs with naturally infected milk*

Milk sample	Number of surviving guinea-pigs			Number of surviving mice			Animals dying
	Infected	Agglutinin titre	not infected	Infected	Agglutinin titre	not infected	
1	1	1 80	0	1	1 40	2	1 guinea pig 1 mouse
2	1	1 640	0	1	1 160	3	1 guinea pig
3	2	1 80 1 320	0	0		4	
4	2	1 2560 1 320	0	1	1 320	1	2 mice
5	2	1 640 1 320	0	0		2	2 "
6	2	1 2560 1 640	0	0		3	1 mouse
7	1	1 40	1	0		4	
8	2	1 640 1 320	0	0		3	1 mouse
9	2	1 320 1 320	0	0		3	1 "
10	2	1 320 1 640	0	0		2	2 mice
Totals	17	.	1	3		27	2 guinea-pigs 10 mice

and 10 mice died after inoculation and in these no post-mortem examination was carried out Eighteen guinea-pigs survived the period of the experiment and *Br. abortus* was recovered from the spleens of 17 of them In the 30 mice surviving and examined, *Br. abortus* was recovered from 3 animals only (samples 1, 2 and 4). In sample 1, 1 mouse out of 3 was infected, in sample 2, 1 mouse out of 4, and in sample 4, 1 mouse out of 2.

*Agglutination titres in mice and guinea-pigs*

The titres in mice infected with culture were as a rule much lower than those in infected guinea-pigs inoculated with the corresponding numbers of organisms (table IV). It will be seen that the titres recorded in expts I, II and III, where strain B 54 was used, were higher in both animals than in expt IV, carried out with strain Ast 370. In mice it would seem that the highest incidence lies between 1/80 and 1/10 with B 54 as against 1/640

TABLE IV

*Agglutination titres in infected mice and guinea pigs*

Titres	Number of mice					Number of guinea pigs		
	Expt I	Expt II	Expt III	Expt IV	Totals	Expt III	Expt IV	Totals
1/640+	2	0	0	0	2	18	0	18
1/320	3	5	0	0	8	2	0	2
1/160	3	2	8	0	11	0	2	2
1/80	3	3	11	2	19	5	6	11
1/40	1	1	15	8	25	3	0	3
1/20	3	3	10	8	34	0	2	2
1/10	2	1	14	2	19	0	2	2
0	1	0	1	11	13	0	0	0
Totals	18	16	65	31		34	12	

or higher in guinea-pigs. With strain Ast 370 the highest incidence was between 1/40 and 0 in mice, and between 1/80 and 1/10 in guinea-pigs. There was no correlation between titre and the number of organisms injected. In 2 infected mice of the B 54 groups (expts I, II and III) and in 11 of the Ast 370 group (expt IV) there were no demonstrable agglutinins in the blood serum. Agglutinins were never detected in mice from which *Br. abortus* could not be isolated from the spleen.

*Discussion*

In this investigation it was found that relatively large doses of two strains of *Br. abortus* were required to infect mice. In a total of 213 mice injected with graded doses of cultures ranging from  $810 \times 10^6$  to 28 organisms, *Br. abortus* was recovered from the spleens of 136 or 63.90 per cent of the animals inoculated. The minimal infective dose with one of the strains was  $280 \times 10^2$  organisms and *Br. abortus* was recovered from approximately only one half of the animals injected with this dose. Injection of much larger numbers of organisms did not result in certain infection—e.g. injection of  $810 \times 10^6$  organisms, the highest dosage used, resulted in infection of 14 out of 19 mice. This is contrary to the findings

of Ber (1933, 1936), who states that he was able to secure infection with 8 or 9 organisms. It is difficult to account for this discrepancy. It is possible that the strains of *Br. abortus* used in our work were less virulent than those used by Ber, but this is a point which would be difficult to establish with organisms which produce a chronic type of disease. The results of Feldman and Olson appear to be more in accordance with those of the present work. For instance, they obtained infection in 28 out of 34 mice with 0.25 c.c. of suspensions of *Br. abortus* (bovine and porcine), comparable to tube 1 of the McFarland nephelometer. This would appear to be approximately equivalent to 75,000 organisms (Kelser, 1933).

Guinea-pigs were more susceptible to infection with our strains than were mice. This was so clear-cut that the use of statistical methods for revealing differences became superfluous. Forty-nine guinea-pigs injected with similar numbers of organisms were examined and of these 46 or 93.88 per cent were found to be infected. As low a dosage as 28 to 280 organisms resulted in infection of 6 out of 9 guinea-pigs.

With the strains used in our experiments no rapidly fatal prostrating disease was observed in mice even after the inoculation of massive doses. The most characteristic macroscopic finding *post mortem* was a very much enlarged and swollen spleen of a deep red colour. In a few animals there were large abscesses in the liver but, as *Br. abortus* could never be recovered from them, no significance was attached to their presence.

The antigenic response of mice to *Br. abortus*, as judged by the agglutination test, appears to be much less than that of guinea-pigs. This is in agreement with the experience of other workers.

Evidence was obtained which showed the undesirability of mice replacing guinea-pigs for the isolation of *Br. abortus* from naturally infected material. Ten samples of milk were found on guinea-pig inoculation to contain *Br. abortus*, whereas in mice inoculated with corresponding material, infection was diagnosed in only 3 of the samples and *Br. abortus* was recovered from only a proportion of the mice in each case.

#### Summary.

In a total of 213 mice injected with graded doses of *Br. abortus* cultures ranging from  $810 \times 10^6$  to 28 organisms, 136 or 63.90 per cent were found to be infected. The smallest number of organisms which resulted in infection was  $280 \times 10^2$ . The highest dose used namely  $810 \times 10^6$  organisms, did not result in infection of all the mice in this group. Forty-nine guinea-pigs injected with corresponding doses of cultures were examined and of these 46 or 93.88 per cent were found to be infected. As low a dosage as 28-280 organisms gave rise to infection in 6 out of 9 guinea-pigs.









reviewing similar evidence, do not think "there is yet any reason for reserving the term 'gravis' for one particular serological type of the organisms so named by McLeod"

*Methods of preparation of antisera and agglutinable suspensions.*

Miss Orr-Ewing very courteously sent me her 4 type strains A, B, C and D, labelled SAS (Leeds), T Douglas (Oxford), M D 13 (Poplar) and Wadi Atali (Khartoum) respectively.

The technique described by Orr-Ewing of inoculating rabbits with cultures suspended in 2 per cent formol-saline and detoxicated for five days at 37° C was found satisfactory. The initial dose was 500 million organisms, which was subsequently increased after 4-5 inoculations to 2500 million. Higher doses were found to be unsuitable. Suspensions were given intravenously at five-day intervals, shortened to four days and three days towards the end of the course, and dosage was varied according to the weight and general condition of the rabbits. Ten to fourteen injections were found necessary to bring the serum to a satisfactory titre of about 1:1600.

Considerable difficulty has been found by many observers in preparing stable suspensions of the *gravis* type of *C. diphtheriae*, but a modification of the method adopted by Orr-Ewing was found to be satisfactory. Cultures were grown on inspissated serum and this seemed to be important, as serum agar produced a very refractory suspension. A 24-hour growth on a 4-inch plate was rubbed up with 2 c.c. of saline and scraped off with a bent glass spreader, the resulting dense suspension being pipetted into glass bottles containing glass beads, heated at 56° C for one hour and then shaken 200-300 times per minute for 15-30 minutes. The suspension, which still contained some large particles, was pipetted into deep tubes of 0.9 per cent saline and allowed to sediment for at least one hour. Sufficient of the homogenous upper layer was then transferred to smaller tubes of saline to make a suspension suitable for agglutination purposes. This suspension was perfectly stable over the 24-48-hour period required for agglutination tests.

Agglutination tests were put up in double dilutions from 1:50 to 1:6400 and read after four hours in the water-bath (56° C) and again after standing at room temperature overnight. Results generally showed in the lower dilutions a coarse semi-flocculent agglutination and in the higher a definite granularity. Control tubes remained unchanged.

*Varieties of starch-fermenting strains*

Two varieties of starch-fermenting strains of *C. diphtheriae* were encountered. These are called, for reasons given later, types A and B (1).

*Morphology.* Original cultures from throat swabs were grown on Loeffler slopes and the morphological features of the diphtheria bacilli found were recorded at the first examination. These showed great variation. Type A might be short, long, stout or slender, and showed barred and beaded appearances in about half the number examined, terminal granules might be marked, moderate or absent, but were generally poorly developed. Very occasionally the organisms were regular and rather like *C. hofmanni*, but they generally showed some degree of pleomorphism and irregularity.



B (1) type. Agglutination of the homologous organisms was distinct and easily read to 1:1600 or 1:3200. A slight degree of cross agglutination at lower titres (1:100 to 1:400) was noticed. These co-agglutinins could easily be absorbed without affecting materially the original titre of the serum but they interfered so little with the test that it was not found necessary to do this as a routine.

Type A (Newcastle) agglutinated to full titre with an antiserum prepared against Orr-Ewing's A type and was otherwise similar, so that one may consider the A type here described to be identical with the original *gravis* type of the Leeds workers. Type B (1) strains agglutinated to full titre with an antiserum to Orr-Ewing's type B and reciprocal absorption tests proved that they were serologically identical. The type B strain supplied by Miss Orr-Ewing produced a typical *gravis* colony and showed pellicle formation and a heavy granular deposit in broth, my type B (1) was consistently distinct from it both morphologically and culturally. These observations have led me to distinguish the Newcastle strain as type B (1).

#### *Serological relationship of other diphtheria and diphtheroid bacilli to gravis strains*

Fourteen 'intermediate' *L. mitis* and 14 atypical strains of *C. diphtheria* as well as 4 strains of *C. hoefmanni* and one saccharose-fermenting diphtheroid were tested against the antisera prepared. No significant agglutination occurred except with certain of the atypical strains. These showed typical daisy-head *gravis*-like colonies and gave pellicles and coarse deposits in broth but did not ferment starch; they appeared to correspond to Wright and Christison's types IV and VI. Most of them were type VI—non-virulent—and were not isolated from clinical diphtheria. Such strains, however, agglutinated nearly to full titre with Orr-Ewing's *gravis* type D antiserum, but were not identical by reciprocal absorption of agglutinin. They absorbed all the D agglutinin from a D antiserum, but a type D strain absorbed only partially the type VI (Wright and Christison) agglutinin from a type VI antiserum.

#### *Clinical findings*

The differences observed in the two types induced me to investigate their clinical importance. During the year ending March 1935, 1607 strains were collected in the routine examination of sputa for the City of Newcastle, of which 116 were from cases of diphtheria, the remainder being from carriers and other sources. All were checked serologically. Through the courtesy



B (1) type Agglutination of the homologous organisms was distinct and easily read to 1 1600 or 1 : 3200 A slight degree of cross agglutination at lower titres (1 100 to 1 400) was noticed These co-agglutinins could easily be absorbed without affecting materially the original titre of the serum, but they interfered so little with the test that it was not found necessary to do this as a routine

Type A (Newcastle) agglutinated to full titre with an antiserum prepared against Orr-Ewing's A type and was otherwise similar, so that one may consider the A type here described to be identical with the original *gravis* type of the Leeds workers Type B (1) strains agglutinated to full titre with an antiserum to Orr-Ewing's type B, and reciprocal absorption tests proved that they were serologically identical The type B strain supplied by Miss Orr-Ewing produced a typical *gravis* colony and showed pellicle formation and a heavy granular deposit in broth, my type B (1) was consistently distinct from it both morphologically and culturally These observations have led me to distinguish the Newcastle strain as type B (1)

#### *Serological relationship of other diphtheria and diphtheroid bacilli to gravis strains*

Fourteen "intermediate," 4 *mitis* and 14 atypical strains of *C diphtheriae* as well as 4 strains of *C hofmanni* and one sucrose-fermenting diphtheroid were tested against the antisera prepared No significant agglutination occurred except with certain of the atypical strains These showed typical daisy-head *gravis*-like colonies and gave pellicles and coarse deposits in broth but did not ferment starch, they appeared to correspond to Wright and Christison's types IV and VI Most of them were type VI—non-virulent—and were not isolated from clinical diphtheria Such strains, however, agglutinated nearly to full titre with Orr-Ewing's *gravis* type D antiserum, but were not identical by reciprocal absorption of agglutinins They absorbed all the D agglutinins from a D antiserum, but a type D strain absorbed only partially the type VI (Wright and Christison) agglutinins from a type VI antiserum

#### *Clinical findings*

The differences observed in these two types induced me to investigate their clinical importance During the year ending March 1935, about 150 strains were collected in the routine examination of swabs for the City of Newcastle, of which 116 were from cases of diphtheria, the remainder being from carriers and other sources All were classified serologically Through the courtesy

viewpoint is also open to criticism, in Newcastle the type B (1) strains associated with mild infections are serologically identical with, though otherwise distinct from, the type B strains which in certain areas produce severe infections (Robinson and Peeney). In my own observations, however, one group of *C. diphtheriae* with the full biological characters of the Leeds *gravis* type shows a case mortality of 13.18 per cent while another group differing in colony appearance from any of the recognised *gravis* types, though serologically identical with one (*gravis* type B), shows a mortality of only 2 per cent. That such strains are fairly widespread is suggested by the fact that Wright and Christison mention three strains of atypical starch-fermenters which resembled their type II ("intermediate"). Mair speaks of a group of barred starch-fermenters, apparently akin to "intermediate," which were serologically identical with Leeds *gravis*, and one or two irregular starch-fermenters, *mitis*-like in culture, which agglutinated to full titre with an antiserum prepared against Orr-Ewing's *gravis*, type B. The necessity of eliminating all such atypical strains in the assessment of the epidemiological importance of McLeod's *gravis* type is evident.

#### Summary

Two distinct types of starch-fermenting diphtheria bacilli occurring in Newcastle have been described. They differed in morphology, in colonial appearance, particularly on chocolate tellurite medium, in growth in broth and in haemolysis on horse blood plates. One was serologically and culturally identical with the Leeds strain called *gravis* A by Orr-Ewing, the other was serologically identical with Orr-Ewing's *gravis* B strain, but was culturally distinct and most resembled "intermediate" strains. It has been tentatively called B (1). Clinically A strains were responsible for a much more severe type of diphtheria than were B (1) strains.

The observations recorded in this paper indicate that dependence on either colonial appearance, starch fermentation or serological typing alone is not sufficient for the diagnosis of the *gravis* group of diphtheria bacilli, and suggest that such a diagnosis should be made only when an organism shows the combined characters of coarse colony morphology, the formation of a pellicle and a granular deposit in broth, and starch fermentation.

The author wishes to thank Dr Charles, Medical Officer of Health for Newcastle, for permission to publish the clinical details included in this paper.

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No cases of infection with type B (1) were found in immunised persons

### Discussion

The classification of diphtheria bacilli is difficult owing to a confusion of terms, and comparison of various workers' findings will on this account shortly be impossible. Biologically McLeod's original *gravis* type is probably approximately the same as Marr's (1936) non-barred starch fermenter and is identical with Wright and Christison's type III. Serologically it is Orr-Ewing's *gravis* type A or Robertson and Peeney's *gravis* type I. As the latter workers suggest, serological types are often best represented by numbers. To avoid subsequent confusion it would be advisable, however, not to classify by means of numbers until the whole group of *C. diphtheriae* can be adequately divided on a serological basis. For practical differentiation at the moment the terms *gravis* and *mitis* are very difficult to better, but considerable clinical confusion may arise if at any time in the future the relative severity of infection due to these strains is altered or even reversed. It seems likely from the observations in this paper that serological types are not necessarily uniform in their disease-producing activities, and consequently that the biological criteria established by McLeod are still of paramount importance. The terms used by him have therefore been retained here, together with (on the ground of priority) the serological groupings of Orr-Ewing, the type B (1) is so named merely for convenience of recognition and no permanence is desired. It is suggested, however, that a much more satisfactory, if less expressive, nomenclature would be achieved by adopting letters for distinct biological groups and numbers for serological types, preferably within the whole group. Thus biological variants of a distinct serological type could readily be distinguished by the serological numeral and the biological letter.

In any investigation into the epidemiological importance of particular strains of diphtheria bacilli, two methods are likely to be employed, the organisms may be classified on the criteria of the Leeds workers or on serological lines. In the former, investigators may be tempted to place undue reliance on individual characteristics such as starch fermentation (Menton, 1932, Christison, 1933), or on roughness of colony morphology (Anderson *et al.*, 1931). That such a procedure is open to grave objection is shown in the series of cases presented here where one group of starch-fermenting *C. diphtheriae* has produced very mild infections. Further it has been shown by Wright and Christison that roughness of colony morphology is not necessarily associated either with starch fermentation or with clinical virulence, and my observations are in entire agreement. Investigation from a purely serological





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halved the concentration of sodium sulphite. This modification is what is referred to throughout this paper as "modified Wilson and Blair medium."

*Bact paratyphosum B* grew as a rule within 18 hours, yielding black colonies 2-3 mm in diameter, flat, glistening and smooth, surrounded by a brown-black halo  $\frac{1}{2}$  in in diameter showing the characteristic metallic sheen. The growth of *Bact coli* and other faecal organisms was more or less inhibited, but even when the plates were overcrowded with colonies of other organisms it has always been possible to detect at once the paratyphoid colonies and to obtain them in pure culture. Later it was found that, by reducing the concentration of anhydrous sodium sulphite to 0.5 per cent, the growth of *Bact coli* was completely inhibited, whereas that of *Bact paratyphosum B* was unimpaired. With *Bact typhosum*, however, our results varied with different batches of the medium and we never obtained the same constancy of results as with *Bact paratyphosum B*. For some unknown reason the same strain of *Bact typhosum* would grow differently not only on batches of medium prepared with two different stock mixtures but even on batches made up with the same stock mixture. We were therefore obliged to investigate the role and importance of each of the constituents of the medium as regards growth, selectivity and blackening.

#### *The role of the various ingredients of Wilson and Blair's medium*

This was investigated by incorporating the various ingredients, alone or in combination, in nutrient agar prepared according to Wright's method (Wright, 1933, 1934), allowing plates thereof to cool and dry and inoculating the surface with a loopful of a suspension of bacteria so adjusted as to give a countable number of colonies on a control agar plate and comparing the results on the test media with suitable controls as regards number, size and differentiation of colonies. The bacterial suspensions were made up in a fluid containing 0.85 per cent NaCl, 0.15 per cent  $\text{Na}_2\text{HPO}_4$  and 0.1 per cent gelatine. Experiments were made with a number of strains of *Bact typhosum*, *Bact paratyphosum B* and *Bact coli*.

**Sodium sulphite.** Up to a concentration of 1.5 per cent in nutrient agar, anhydrous sodium sulphite alone had no inhibitory action on the organisms tested. With a concentration of 2 per cent the size of the colonies was reduced but this reduction was equally marked with all.

**Glucose.** Without glucose *Bact typhosum* and *Bact paratyphosum B* were unable to produce  $\text{H}_2\text{S}$  from sodium sulphite. It was also observed that blackening did not readily occur in media in which large amounts of acid were produced.

(p. 376) to be to "absorb the acids produced in the fermentation of glucose and ensure the blackening of the colony with iron sulphide." It would appear that they believed that the black pigment produced was iron sulphide and that the bismuth acted as a selective agent in conjunction with sodium sulphite. Brilliant green was added because it enhanced the selectivity of the medium. In 1928 Wilson recorded the isolation of *Bact. typhosum* on such a medium from sewage and shellfish. In 1931 Wilson and Blair described three varieties of media. Of these, one contains bismuth, sulphite, glucose phosphate and iron and the other the same ingredients with the addition of brilliant green. These two media are known as "Old standard medium" and the concentrations of the ingredients are identical with those reported in 1933 (Wilson, 1933) and given in detail below. They found that it was possible to obtain black surface colonies of *Bact. typhosum* on a medium free from iron. This new medium they called "New standard bismuth sulphite brilliant green medium." It is an important feature of this modification that, with the exception of brilliant green, the concentrations of the ingredients were half those of the old standard medium. They made the very important statement that *Bact. paratyphosum B* grew much better when the amount of sulphite used was three-fifths of that recommended for the typhoid bacillus.

In 1933 Wilson introduced the use of bismuth ammonio citrate scales instead of liquor bismuthi et ammonii citratis, which considerably simplified preparation. The method which he finally arrived at is the following (p. 561). "Dissolve 6 grams bismuth-ammonio-citrate scales in 50 c.c.m. boiling distilled water, and neutralize by the addition of about 2 c.c.m. of 10 per cent caustic soda. Mix with a solution obtained by boiling 20 grams sodium sulphite anhydrous in 100 c.c.m. of water, and then, while the mixture is boiling, add 10 grams of sodium phosphate anhydrous. To the sulphite-bismuth-phosphate mixture, when cool, add a solution of glucose obtained by dissolving 10 grams of commercial glucose in 50 c.c.m. of boiling distilled water. For preparation of old standard medium, add to 100 c.c.m. of a hot melted 3 per cent nutrient agar 20 c.c.m. of stock mixture, then 1 c.c.m. of an 8 per cent solution of ferrous sulphate crystals in water, and 0.5 c.c.m. of a 1 per cent solution of brilliant green in distilled water. Pour into Petri dishes, and when the medium has set inoculate the surface."

In this medium the concentrations of the ingredients in 100 c.c. are approximately as follows:

Bismuth ammonio-citrate	0.6 g
Anhydrous sodium sulphite	2.0 g
Anhydrous sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )	1.0 g
Glucose	1.0 g
Ferrous sulphate	0.08 g

The final concentration of brilliant green is 1:20,000. In our hands the reaction has usually been pH 8.1-8.2.

During an epidemic of paratyphoid B fever which broke out in Liverpool at the beginning of January 1937 we used with great success a modification of Wilson and Blair's medium for the isolation of *Bact. paratyphosum B* from the faeces. Following the suggestion of Wilson and Blair (1931) we replaced the anhydrous sodium sulphite by the crystalline salt. Since the molecular weight of anhydrous sulphite is 126 and that of the crystalline form 252, we simply

produced by this concentration of bismuth ammonio citrate either alone or in combination with sodium sulphate was not specially directed towards *Bact coli*

Our experiments indicate that the blackening of the medium and of the colonies with *Bact typhosum* and *Bact paratyphosum B* is mainly dependent on the presence of bismuth. When plates of modified Wilson and Blair medium from which bismuth ammonio-citrate was excluded were inoculated with *Bact typhosum* or *Bact paratyphosum B*, sulphide was produced in great amount, easily detected by lead acetate papers, but the colonies either remained white or showed a grey central spot. As iron was present in the usual concentration it seems evident that it is not responsible for the blackening. That it has nothing to do with the blackening is more difficult to prove, for, when ferrous sulphate is not incorporated in the medium, both organisms fail to grow.

Two experiments, however, enabled us to show beyond doubt that iron is not necessary for the blackening and that the black pigment produced is a bismuth compound.

**Experiment I** Two plates of modified Wilson and Blair medium from which ferrous sulphate and brilliant green were excluded were inoculated with *Bact paratyphosum B*. Ten minutes later a layer of the same medium cooled down to 45° C was poured over one of the plates (plate A), whereas the other was covered with a layer of nutrient agar (plate B). As controls a plate of the same bismuth medium (plate C) and one of nutrient agar (plate D) were similarly inoculated but were not covered with a second layer. No iron was added to any of the plates. After 20 hours' incubation, colonies could be seen in the depth of plate B (bismuth medium covered with agar) and at the surface of plate D (nutrient agar). There was no growth on either A or C. After 36 hours there was still no growth on A and C, whereas a deep brown halo had developed round a great number of the colonies in B. When the two layers of this medium were separated, the colonies had a tendency to stick to the nutrient agar, leaving a small pit in the Wilson and Blair layer. Round this tiny pit a typical halo could be seen, showing that although the colonies developed in the agar layer the halo was actually in the bismuth medium and as this contained no added iron the blackening was presumably due to the action of sulphide on the bismuth.

**Experiment II** Two plates A and B were divided across one diameter by a vertical partition. Modified Wilson and Blair medium without iron and brilliant green was poured in side "a" of both plates. The medium was allowed to set and the partitions were removed. Side "b" of plate A was filled with modified Wilson and Blair medium from which bismuth and brilliant green were excluded, side "b" of plate B with the same medium from which bismuth, iron and brilliant green were excluded. Side "b" of each plate was inoculated with *Bact paratyphosum B* along a line parallel to the partition and at a distance of half an inch from it. After incubation for 18-20 hours the colonies which had developed were white, both in the plate which contained iron and in the one which did not, bismuth being absent from both, whereas the uninoculated sides (a) of both plates showed the characteristic blackening. As the uninoculated sides contained bismuth but no iron it is clear that the blackening was due to the action upon the bismuth of sulphide which had diffused from the inoculated side of the medium.

*Bismuth ammonio-citrate* (scales, B D H) On nutrient agar containing this salt in a concentration of 0.6 per cent (pH 7.1), *Bact typhosum* produced pin-point colonies while the colonies of *Bact paratyphosum* B and *Bact coli* did not exceed 1 mm. in diameter. Adjusting the pH to 7.8 did not affect the result. When caustic soda was added by the same technique and in the same concentration as for the preparation of the stock mixture, the reaction of the medium was pH 7.3 and the result unaltered. Again adjusting the pH to 7.7 was without effect.

The slight inhibitory effect of this concentration of the bismuth salt (either with or without NaOH) was considerably enhanced by the addition of 1 per cent anhydrous sodium sulphite. Small inocula (30-50 organisms) of *Bact typhosum* and *Bact paratyphosum* B failed entirely to grow in 48 hours and *Bact coli* was considerably delayed. When 0.08 per cent ferrous sulphate was added all three organisms grew well, the paratyphoid B bacilli and *Bact coli* within 18 hours and the typhoid bacilli within 22. We are unable to define the nature of this inhibition, but there is little doubt that it is due to some soluble form of bismuth.

Six g of bismuth ammonio-citrate were dissolved in 50 c.c. of boiling distilled water and 1 c.c. of 20 per cent NaOH added immediately. A white precipitate formed and the mixture was allowed to cool. Ten g of anhydrous sodium sulphite were dissolved in 100 c.c. of boiling distilled water, cooled and then added to the bismuth preparation. The precipitate dissolved, but on heating, a fresh heavy white precipitate formed and rapidly deposited, leaving a clear supernatant fluid. This fluid contains a very small amount of bismuth, most probably in solution. In agar media the supernatant fluid showed practically the same inhibitory properties after incubation for 24 hours at 37° C. as the mixture of precipitate and supernatant and in each case the inhibition was abolished by the addition of ferrous sulphate (0.08 per cent).

In another experiment 4.5 c.c. of the mixture of precipitate and supernatant fluid were added to each of a series of flasks containing 30 c.c. of nutrient agar. The contents of each flask were poured into a Petri dish and the medium allowed to set. Ten c.c. of melted nutrient agar were then poured on top of the first layer and allowed to set. The plates were stored in the dark for 24-48 hours and then inoculated with *Bact typhosum*, *Bact paratyphosum* B and *Bact coli* and incubated. Another series of plates each containing 40 c.c. of nutrient agar and 4.5 c.c. of the mixture but without any covering layer of nutrient agar were stored and inoculated in the same way. Both sets of media showed the same marked inhibitory effect, from which it appears necessary to conclude that the inhibitory factor is a soluble substance, able to diffuse through a certain thickness of agar. The inhibition



*Ferrous sulphate* Roughly speaking when no iron is added neither *Bact typhosum* nor *Bact paratyphosum B* will grow either on bismuth-sulphite agar or on the finished Wilson and Blair medium. Nor could we find that it contributed anything to the selectivity of the medium. It is not responsible for the blackening which occurs but, as indicated in the previous section, it does enable the organisms of the typhoid-paratyphoid group to grow on a medium which contains inhibitory concentrations of the bismuth-sulphite mixture.

*Brilliant green* Our experiments indicate that Wilson and Blair's medium owes its selectivity to brilliant green. About 100-200 organisms of each of 4 strains of *Bact typhosum*, 5 strains of *Bact paratyphosum B* and 10 strains of faecal *Bact coli* were inoculated on the six following media.

- (a) Old standard medium
- (b) Old standard medium without brilliant green
- (c) Old standard medium without ferrous sulphate and brilliant green
- (d) Modified Wilson and Blair medium
- (e) Modified Wilson and Blair medium without brilliant green
- (f) Modified Wilson and Blair medium without ferrous sulphate and brilliant green.

On media (c) and (f), to which no iron nor brilliant green was added, all the strains of *Bact typhosum* and *Bact paratyphosum B* failed to grow even after 93 hours' incubation. *Bact coli* grew as a rule but its growth was considerably delayed.

On media (b) and (e), which contained ferrous sulphate but no brilliant green, there was in general more growth, but they still showed some inhibition as compared with the agar controls. On medium (b) 9 out of 10 strains of *Bact coli* gave well defined colonies in 24 hours, the tenth being inhibited, one strain of *Bact typhosum* grew well in 24 hours, 3 others being delayed till 30 hours, all 5 strains of *Bact paratyphosum B* required 36 hours' incubation. On medium (e) none of the strains of *Bact coli* were inhibited and the typhoid bacilli grew as on medium (b), but all the strains of *Bact paratyphosum B* were fully grown in 18 hours. Thus neither on the old standard medium nor on the modification which we have adopted was there any evidence of selectivity in the absence of brilliant green.

With the media which contained brilliant green, (a) and (d), the picture was quite different. On the original Wilson and Blair medium (a) all the strains of *Bact coli* tested failed to grow in 23 hours, but 7 out of 10 appeared in 36 hours, none of the typhoid strains appeared in 24 hours, one was well developed in 28 hours and the others in 36 hours, but 2 showed diminution in the number

of colonies as compared with the control and behaved irregularly on different batches, the paratyphoid B strains showed no growth until after 45 hours. Medium (d), containing half the amount of sodium sulphite present in (a), yielded small colonies of *Bact coli* in 18 hours while the paratyphoid cultures were well developed in this time and the typhoid strains behaved much as on medium A. While, therefore, this medium was satisfactory for paratyphoid B, which it permitted to develop in full as black colonies with a black halo at a time when *Bact coli* was still absent or its colonies small, it was of little value for the typhoid bacillus, which was not well developed until many of the strains of *Bact coli* were also well grown. These results have also been confirmed by field observations on faeces.

*Effect of varying the concentration of bismuth ammonio-citrate and of sodium sulphite* The experiments described above seemed to us to indicate that the selective nature of the Wilson and Blair medium and its modification was entirely due to the brilliant green. It seemed possible, however, that by varying the concentration of the bismuth ammonio-citrate and the sodium sulphite we might find a medium which would exhibit more selective properties and on which *Bact typhosum* would grow more quickly. To that end two block experiments were carried out with media in which the concentrations of sodium phosphate, glucose and ferrous sulphate were kept constant while those of bismuth ammonio-citrate and sodium sulphite were varied, the bismuth salt up to 1.2 per cent and the sodium sulphite up to 2 per cent. Twenty-five media were thus prepared (table). In one experiment brilliant

TABLE  
Concentration of bismuth ammonio citrate and sodium sulphite  
and pH of 25 media used

Anhydrous sodium sulphite (per cent)	Bismuth ammonio-citrate (per cent)				
	0.0	0.3	0.6	0.9	1.2
0.0	A 8.0*	F 7.8	K 7.7	P 7.7	U 7.6
0.5	B 8.0	G 7.0	L 7.9	Q 7.8	V 7.8
1.0	C 8.1	H 8.0	M 8.0	R 7.9	W 7.9
1.5	D 8.2	I 1.8	N 8.1	S 8.0	X 8.0
2.0	E 8.2	J 8.1	O 8.1	T 8.0	Y 8.0

\* Numbers indicate the pH. Letters give reference identifications as used in the text.

green was added in a concentration of 1:20,000, in the other it was omitted. Plates were implanted with small inocula of two strains of *Bact typhosum*, one strain of *Bact paratyphosum B* and one of *Bact coli* and examined after 18, 23 and 37 hours.

**Experiment I** The results on media without brilliant green may be summarised as follows.

In the absence of sodium sulphite (media A, F, K, P, U), no



inhibitory effect was detected. All 4 strains grew as well as on control agar, without any blackening either on or round the colonies.

When the bismuth salt was excluded (media A, B, C, D-E), all 4 strains grew but the size of the colonies became smaller when the concentration of sodium sulphite was 2 per cent.

When both bismuth ammonio-citrate and sodium sulphite were present, different degrees of inhibition were met with, but as a rule *Bact coli* grew better than either *Bact typhosum* or *Bact paratyphosum B*. Medium Y (containing 1.2 per cent of bismuth ammonio-citrate and 2 per cent. of  $\text{Na}_2\text{SO}_3$ ), however, showed a certain selectivity in that *Bact coli* did not grow in less than 30 hours and even after 37 hours' incubation the colonies were smaller than those of the two typhoid strains, which were typical at the end of 34 hours incubation. *Bact. paratyphosum B* grew badly on medium Y and best on medium L (bismuth salt 0.6 per cent, sulphite 0.5 per cent). Increasing the concentration of the bismuth salt and the sulphite made the medium worse for the paratyphoid and colon bacilli but had less effect on the growth of *Bact typhosum*, though its growth was still rather slow.

It seemed possible that further increase in the concentration of the salts in question might produce an even more selective medium for typhoid. The use of 1.5 per cent of bismuth ammonio-citrate and 3 per cent of sodium sulphite promised to provide the desired result, but further experience with a variety of strains gave results which were too irregular to be of any value. It is of some interest that, whereas we have found that *Bact paratyphosum B* is in general a hardier organism and more resistant to inhibitory influences than *Bact typhosum*, in this particular instance the reverse is true.

**Experiment II** The results in the experiment in which brilliant green was incorporated in the media at a concentration of 1 : 20,000 were as follows.

On media A, F, K, P, U (increasing concentrations of bismuth) *Bact typhosum* and *Bact coli* did not grow at all, but *Bact paratyphosum B* grew well on media A, F and K, badly on medium P and not at all on medium U. This indicates that brilliant green is less inhibitory for *Bact paratyphosum B* than for the other two organisms and its effect is increased in the presence of the higher bismuth concentrations.

Of the media containing no bismuth but increasing amounts of sulphite, only *Bact paratyphosum B* grew on B, while on C, D and E all three types flourished, from which it would appear that sodium sulphite decreases the bacterostatic action of brilliant green.

On the other media of the series, which contained both bismuth

and sulphite in varying proportions, *Bact paratyphosum* B was well developed within 18 hours on media G, H, L and M. In the same time *Bact coli* was fully grown on G and H, much less fully on M and not at all on L. Medium M corresponds to the modified Wilson and Blair medium as defined previously. On I, J, N, R and V the paratyphoid bacillus grew poorly in 18 hours and on O (the original Wilson and Blair medium), S, T, W, X and Y no growth occurred in this time. On many of these media it produced characteristic colonies after longer incubation. On none of them was the growth of the typhoid bacillus characteristic until after more than 24 hours' incubation. At 36 hours the growth was typical and equivalent to that on the controls on H, I, M and N. Media M and N gave less growth of *Bact coli* at this time than did H and I. Media G, J, O, R, S, W and X gave fair growth, but T and Y only very little. *Bact coli* did not grow in 36 hours on Q, R, S, T, V, W, X or Y, grew poorly on L, M, N and O and freely on G, H, I and J.

It thus appeared that brilliant green did confer selectivity on the medium, that medium L was a satisfactory one for early isolation of paratyphoid bacilli but that none of the series was suitable for the early isolation of the typhoid bacillus. Strains showed considerable variation in their behaviour. Media M and N permitted isolation of some strains at 24 hours but others failed to appear until later and were not quantitatively recovered. It remained to consider the concentration of phosphate.

*Sodium phosphate* ( $\text{Na}_2\text{HPO}_4$ ). In Wilson and Blair's medium the concentration of this salt is 1 per cent. We have found that even 2 per cent does not produce any inhibition of the *coli*-typhoid group in ordinary agar. In the course of some experiments with a particularly delicate and troublesome strain of *Bact typhosum* we made an observation which suggested that reduction of the phosphate might be advantageous. Four batches of the modified Wilson and Blair medium were prepared, one without addition of sodium phosphate and the other three with 0.25, 0.5 and 1 per cent of that salt respectively. These were inoculated with measured small inocula of a delicate and a relatively resistant strain of *Bact typhosum* and a delicate and a resistant strain of *Bact coli*. Without phosphate no blackening occurred or else it was minimal, varying with the batch of agar used and, as we think probable, with its phosphato content. The most satisfactory concentration proved to be 0.25 per cent. On the medium containing this amount the resistant typhoid strain was fully developed and typical in 18 hours, the delicate strain in 22 hours, the strains of *Bact coli* appeared in 22 hours as tiny colourless colonies which could very easily be distinguished. These results we confirmed in experiments with 10 strains of *Bact typhosum* and 10 strains of *Bact coli*. The

inhibitory effect was detected. All 4 strains grew as well as on control agar, without any blackening either on or round the colonies.

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On the other media of the series, which contained both bismuth



medium was also found to be very satisfactory for the paratyphoid B bacilli although the blackening was perhaps less intense than on our usual medium. The explanation of the harmful effect of excess of phosphate seems to us to lie in the interaction between sodium phosphate and ferrous sulphate. We have seen that, in order to obtain growth on bismuth-sulphite agar, the addition of iron was necessary. If to this bismuth-sulphite-ferrous sulphate agar 1 per cent of sodium phosphate is added the medium becomes too inhibitory for some strains of *Bact typhosum*. This drawback could be overcome by increasing the amount of iron. It seems that an optimal ratio iron/phosphate is desirable to ensure good growth. That is to say, more iron should be added to the medium if the concentration of phosphate is to be kept at 1 per cent, or, if the concentration of iron is not to be raised above 0.08 per cent, the amount of phosphate should be decreased. We have adopted the latter alternative as excess of iron gives to the medium a soft consistency. In its effect on the blackening of the medium sodium phosphate apparently acts as a buffer. It seems that, below a certain pH, *Bact typhosum* and *Bact paratyphosum B* are unable to reduce sulphites to sulphides. We found that the production of sulphide, as judged by colonial appearance and tests with lead acetate paper, was more intense and lasted longer in those plates where the concentration of phosphate was high. What we have to aim at is a concentration which favours both blackening and growth and this seems to be best provided at 0.35 per cent.

### Discussion

In all investigations of this sort it is important to bear in mind that, just as there are differences between groups of bacteria in relation to their susceptibility to growth inhibition, so there may be differences between individual members of a group. This has been forcibly brought to our notice in testing different strains of *Bact. typhosum* and *Bact coli* though it has been less evident with paratyphoid bacilli. We have attempted to eliminate this kind of complication by testing a variety of strains of varying susceptibility and by a limited application of our conclusions to routine work by the use of a medium devised in accordance with our experience. We are, however, alive to the possibility that there may be still more fastidious strains of typhoid bacilli and more resistant strains of *Bact coli* than those we have used, to which these conclusions may not apply.

It would appear that none of the ingredients recommended by Wilson and Blair may be omitted without impairing the medium. If, however, a medium is to be constructed which is equally suitable for typhoid and paratyphoid bacilli and sufficiently selective and differential, the proportions of these ingredients require some



### Summary

1 The selectivity of Wilson and Blair's medium is largely dependent on the use of brilliant green. In relatively high concentrations bismuth ammonio-citrate and sodium sulphite show slight selective action for *Bact typhosum*.

2 The characteristic blackening is due to formation of bismuth sulphide. This does not occur in the absence of glucose and is aided by ferrous sulphate, which affects the amount of growth, and by phosphates, which buffer the medium.

3 Excess of sodium sulphite interferes with the growth of *Bact paratyphosum* B and excess of sodium phosphate with that of some strains of *Bact typhosum*.

4 A medium is described which embodies these findings and is equally suitable for the cultivation of typhoid and paratyphoid B bacilli.

It is a pleasure to acknowledge the kind assistance of Professor W J Wilson, who placed at my disposal some of his own stock solution and completed medium for comparison with our own and gave valuable advice on certain points, and also of Professor H D Wright and Dr V. Glass.

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" "		1933	<i>Ibid</i> , ii 560
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### Appendix

#### Preparation of the medium

*Stock mixture.* 1 Label 3 sterile flasks A, B and C and place in flask A 6 g bismuth ammonio-citrate (scales, B.D.H.), in flask B, 10 g sodium sulphite anhydrous, in flask C 10 g glucose.

2 To flask A add 50 c.c. boiling distilled water and shake until the bismuth ammonio-citrate is dissolved. add 1 c.c. 5N NaOH, mix well and allow to cool. To flask B add 100 c.c. boiling distilled water and allow to cool. To flask C add 50 c.c. cold distilled water. boil 1 minute and allow to cool.

3 Mix the contents of flasks A and B, add 3.5 g sodium phosphate anhydrous ( $\text{Na}_2\text{HPO}_4$ ), bring to the boil, maintain at this point about 30 seconds and allow to cool. add the contents of flask C. This stock









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 E M McV

616—006.1:616 681

## A TERATOMA OF A HORSE'S TESTIS.

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On gelding a horse between eighteen months and two years old, it was found that while one testis was normal, the other was decidedly enlarged

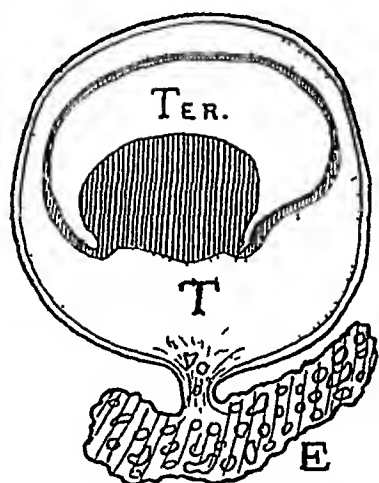


FIG 1—Diagram of a cross section of the testis E, epididymis, T, testis, Ter, teratoma with cyst and mass of solid tissue Natural size

Incision of the enlarged testis disclosed within it a cyst 3.5 cm in diameter containing clear fluid and into which projected a smooth hard mound of tissue 2.5 cm in vertical length, 1.7 cm wide and 1.3 cm in maximum thickness. Testicular tissue surrounded the cyst wall and the base of the solid eminence on all sides (fig 1). The mass and adjacent parts of the cyst wall were cut at right angles to the long axis of the mass into 5 slices each 5 mm thick, from which microscopical sections were prepared.

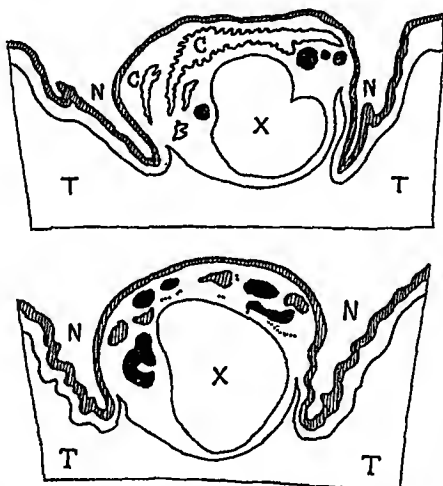
Figs 2 and 3 represent 2 of the 5 section planes and show all the component tissues of the teratoma. All these were fully mature in type. The main cyst was lined everywhere by a layer of neuroglial tissue which in places showed a distinct ependymal surface. The solid mass of tissue contained the following components: (a) patches of neuroglial tissue, some at least of which were continuous with that clothing the surface

of the mass; (b) irregular clefts lined by choroid plexus and communicating at some points with the cavity of the main cyst; (c) some small nerves; (d) a cyst (X) lined by a very thin layer of stratified but non-keratinising epithelium which was pigmented in places; (e) nodules of cartilage; (f) adipose and connective tissue and a few small blood vessels. The growth was fairly well demarcated from the surrounding testis but not separated from it by a capsule.

*Comment.*

Teratomas of the horse's testis are well known, and the object of this report is not merely to record another example of the disease but also to draw attention to certain structural features which resemble those seen in human teratomas.

In a previous paper (Willis, 1937) I decried the use of the name "dormoid" as applied to benign cystic teratomas, all of which contain other tissues besides skin and in some of which even the main cyst is not skin lined. In the present specimen, as in nos XI, XIV and XXI of my previous papers (1935, 1937), the main cyst was lined by nervous tissue and there is no doubt that its watery content was secreted by choroid plexus and was of the nature of cerebrospinal fluid. In this connection, it is noteworthy that of the teratomas so far examined only those which contained choroid plexus, namely nos XI, XII, XIII, XIV, XXI, XXIV and XXVIII of my previous studies and the specimen just described, showed ependyma lined cavities



FIGS 2 and 3—Diagrams of two sections of the teratoma. Hatched areas NN, central nervous tissue, CC, clefts lined by choroid plexus. Dotted lines denote nerves, black areas cartilage, and wide stippling adipose and connective tissue. X, epithelial cyst. TT, testis.  $\times 2$

in their masses of nervous tissue. It appears clear then that these cavities must result from the secretory activity of the choroid plexus tissue.

Another noteworthy feature well seen in figs 2 and 3 is the association of nervous tissue and cartilage, a feature which was observed frequently in my series of human teratomas. In the present growth besides connective tissues and the epithelium of the cyst X, nervous tissues and cartilage are the only components, other tissues which are commonly accompanied by cartilage or bone, such as teeth or respiratory epithelium, are not present. The specimen thus affords additional evidence for the belief that developing nervous tissue can induce cartilage formation in adjacent mesenchyme.

Finally, the diagrams show clearly enough the absence of forthrightness in the teratoma.

*Summary*

A benign teratoma of a horse's testis is described. It exhibited some noteworthy structural features similar to those of human teratomas, namely non-foetiformity, close association of nervous tissue and cartilage, and the presence of a large ependyma-lined cavity in the nervous tissue containing fluid secreted by choroid plexus.

I am indebted to Dr H B Rudduck of Melbourne who kindly gave me this specimen.

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611.018.46:578.65

A STAINING METHOD FOR BONE MARROW FILMS WET  
FIXATION WITH LEISHMAN'S FLUID AND STAINING IN A  
SOLUTION OF ELECTRICALLY CONTROLLED pH \*

PAUL BROWNING.

*From the Pathology Department, University and  
Western Infirmary, Glasgow.*

The following method has been devised in order to overcome some of the difficulties in obtaining satisfactory Romanowsky-stained smears of bone marrow. It has two essential points: (1) the film, before drying has occurred, is fixed with undiluted Leishman's solution—the alcoholic stain when used as the fixative appears to have a mordant-like action on the cells, for it was found that sharper staining was obtained afterwards than when methyl alcohol or any other fixative was used; (2) during staining in the diluted solution a direct electric current is allowed to flow through the staining bath, so that the film is subjected in its different parts to a graded pH. By this means it is possible to prepare films with a very small quantity of marrow, as from a mouse, and the application to human sternal puncture biopsy material is apparent. The extremely clear granular and nuclear differentiation of the various constituents is a feature. The method is equally applicable to human marrow and to that from the ordinary laboratory animals—guinea-pig, rabbit, mouse. Also the varying pH of marrow obtained *post mortem* and other factors which may influence the optimum reaction at which staining occurs need not be taken into account, so that the treatment of a considerable number of smears with buffered solutions of different pH can be obviated. Sometimes partial lysis of the red corpuscles occurs. The circumstances determining this have not been fully ascertained, but pathological conditions of the marrow or *post-mortem* changes appear to be a factor. Other constituents of the marrow are not affected.

*Spreading and fixation of the film.* The film is spread over the middle two-thirds of a slide, either with a second slide, as in the making of a blood film, if the marrow is sufficiently fluid, or better by drawing a piece of tissue along the slide. The film is immediately flooded with Leishman's solution before there has been time for drying. Fixation is allowed to take place

\* This work was done during the tenure of a Coats and Perman Scholarship and I am indebted to the Rankin Fund for a grant toward the expenses of the work.

for one and a half minutes and the stain is then poured off. Taking care that the film does not become dry, one then places the slide face downwards in the staining bath. Staining is effected with a freshly made mixture of one part distilled water and two parts Leishman's stain. During the process a direct electric current is passed through the staining bath. It was found that in this way staining at every pH from highly acid (pH 4) to highly alkaline (pH 11) could be obtained in one film.

**Staining bath.** The shape and dimensions of the staining bath are important. This is a depression in paraffin wax made as follows. A 4 inch Petri plate is filled with melted paraffin wax as used for embedding, which is allowed to set. A glass microscope slide two and a half inches long, made by cutting an ordinary slide, is then placed in the centre of the wax and at either end an aluminium electrode E, out out of 1/32 inch thick sheet aluminium with scissors and of the shape shown in the figure, is fixed into position by heating till its edge (e) is flush with the upper surface of the slide. A full length slide is superimposed on the lower, but overlapping equally at either end. The whole apparatus is then placed in the embedding oven with the door partly open till the wax is just plastic; the slides are firmly pressed down into the wax and the latter is pinched up slightly round the edges of the slides to form a projecting rim. When the wax is nearly set the upper slide is freed by passing the tip of a scalpel round it and prised out, the lower one is removed similarly. The depressions on either side of the staining trough are filled up with molten wax, and when the whole is set a bunsen flame is played lightly over the surfaces so as to fuse them together. The mould which accommodated the upper slide is enlarged slightly both in length and width, so that the slide which is to be stained may slip in easily, and also for another reason mentioned later. It was found that, in order to diffuse the fluid currents set up during staining, the floor of the trough should have a series of grooves. Accordingly about 20 fine grooves (g) equally spaced, 1/32 inch deep, were cut transversely with the tip of a scalpel. The electrodes must be cleared of wax along their parallel surfaces down to the level of the floor of the bath.

The direct current used is a 220 250 volts lighting supply in series with an incandescent electric lamp. The exact rating of the lamp is immaterial in view of the disproportionately high resistance of the bath. The current passed is low,  $\frac{1}{2}$  1½ milliamperes, so that a small wireless valve rectifier\* may be used in the case of A.C. mains. The apparatus can be tested by filling the bath with water containing a few drops of B.D.H. universal indicator. The time should be noted which elapses after turning on the current until the red and blue zones have advanced about one inch from either end. A well graded appearance is presented from acid at one end, through neutral in the centre, to alkaline at the other. This represents approximately the time the current should be allowed to pass during staining, although conditions are slightly different in actual staining owing to the higher conductivity of the marrow and to the diffusion which takes place over its surface. The times mentioned here are those found best for the particular bath, current supply and specimen of Leishman's stain used. Once these times have been fixed by trial, the procedure requires no further modification.

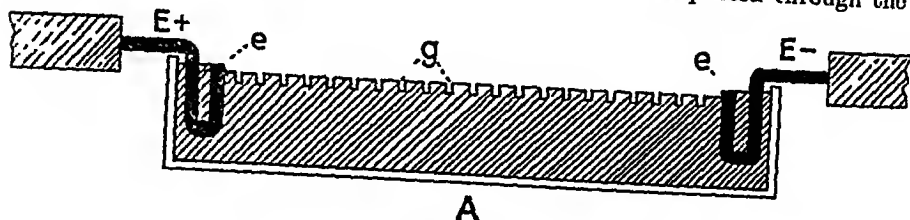
With the slide *in situ*, face down in the staining bath, the current is allowed to pass for three and a half minutes. The slide is then removed and wiped dry around the edges and the extreme ends wiped off if they appear bright red or blue, also any thick parts. Dehydration and differentiation are carried out by running in 1½ per cent coloplionium in absolute alcohol. This

\* This can be made up by a supplier of wireless apparatus if the voltage and milliamperage are specified.

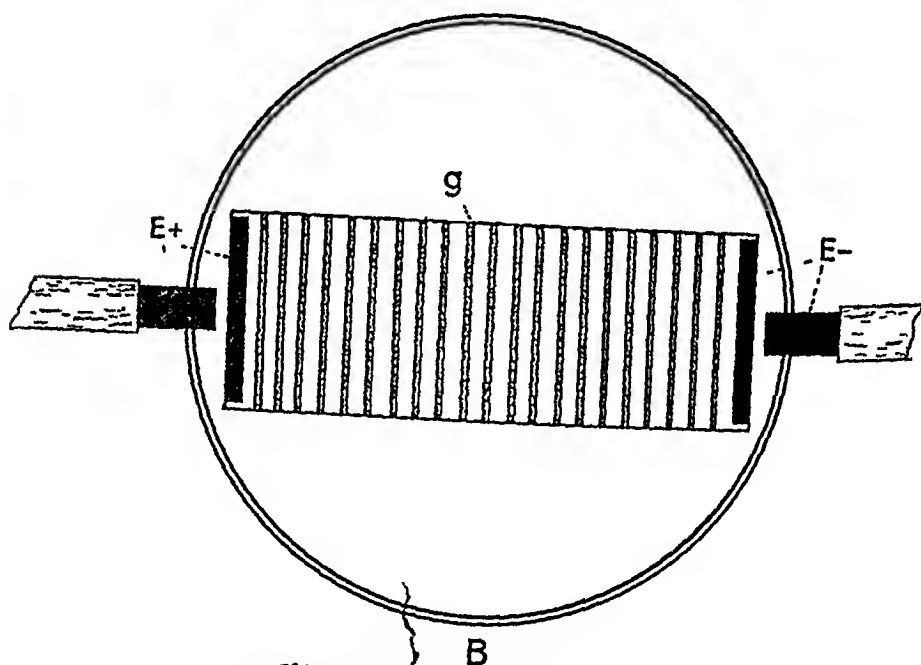
must be done very rapidly to avoid removing the polychrome blues—within not more than a few seconds. The smear is then rinsed in xylol and, if it appears properly dehydrated, mounted in balsam.

In the above case staining was optimal at the time when the current was switched off, but sometimes it is improved by leaving the smear in the staining bath after the current has ceased to pass, *e.g.* for half-a-minute up to several minutes.

Not more than three films should be stained in one filling of the bath, since precipitation occurs when the electric current has passed through the



A



B

considerable ing trough in section.  
biated ing trough viewed from above

occurs in circumstances deter-  
but pathological conditions of the  
to be a factor. Other constituent  
between the staining of each film the contents  
with a pipette to restore the neutral reaction  
Spreading and fixation of the film must be emphasised, since they are essential  
two thirds of a slide, either with a (1) Only the best specimens of Leishman's  
film, if the marrow is sufficiently fluid with ordinary blood films. Owing to the  
ing the slide. The film is immediately important (2) The time allowed for  
there has been time for drying, on treating the stained smear with  
work was done during the time is completed as quickly as dehydration  
applied to the Rankin I used for a. Should any small thick parts of the  
ought to be scraped off after it has been

transferred to xylol (3) The depth of the staining trough must not exceed 1/16 inch, otherwise the transverse grooves are insufficient to diffuse the fluid current properly If there is a tendency for a sharp division to occur between acid and alkaline instead of a gradation over a considerable length of the slide, this can usually be corrected by interrupting the current during staining e.g. by switching the current on and off for ½ minute periods respectively during the staining time of three and a half minutes Alternatively the slide may be slipped gently backwards and forwards in its width to help diffusion Whichever method is adopted, preliminary trials should be carried out with the aid of the universal indicator

Owing to the persistence over long periods of the pH effects established in the trough once the current has been passed for the requisite time and then shut off, staining may be carried out with a dilute solution of the stain over periods up to 18 hours Under these conditions evaporation must be prevented, e.g. by placing a loose Petri lid on the top under which a moist atmosphere is maintained by means of strips of cotton wool steeped in water One advantage of prolonged staining is that the polychrome blues are less easily washed out during dehydration

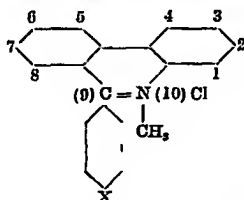
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#### THE TRYPANOCIDAL ACTION OF CERTAIN PHENANTHRIDINIUM COMPOUNDS\*

C H BROWNING, G T MORGAN, J V M ROBB and L P WALLS

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The investigation for chemotherapeutic properties of a series of phenanthridine and phenanthridinium compounds synthesised by two of us (G T M and L P W) has shown that the following possess curative action in mice experimentally infected with trypanosomes



A 7 amino 9 (p aminophenyl) 10 methyl phenanthridinium chloride (NH<sub>2</sub> groups at positions 7 and X)

B 3 (acetylamino) 9 (p acetylaminophenyl) 10 methyl phenanthridinium chloride (NH COCH<sub>3</sub> groups at positions 3 and X)

Both of these substances in doses approaching the maximum tolerated (one subcutaneous injection of 1 mg of A or 2.55 mg of B for a mouse

\* Work done with the support of the Medical Research Council



weighing 20 g) are curative for mice infected with *T. brucei* (strain Paris III—Browning and Gulbrandsen, 1934) when administered 24 hours after inoculation. In addition, with two strains of *T. congolense* in mice (Browning, Cappell and Gulbrandsen, 1934) the 7-amino-9-(*p*-aminophenyl) compound has a curative action on infections. It is of interest that on *Sp. minus* in mice (Browning and Gulbrandsen, 1936) the 3-(acetyl-amino)-9-(*p*-acetylaminophenyl) compound, which is without effect on the latter trypanosomes, has a marked therapeutic effect, although the largest doses do not lead to cure. Since the range of action of these compounds is small, it is not possible to make extensive comparisons between them and analogous substances as regards the relations between chemical constitution and chemotherapeutic action. But the following observations on *T. brucei* infections may be recorded: (1) acetylation of the two  $\text{NH}_2$  groups in compound A results in loss of activity (dose 1.25 mg), (2) the phenanthridine compounds (sulphates) corresponding to A (dose 1 mg) and to B (dose 3.3 mg)—i.e. with H attached to N instead of the  $\text{CH}_3$  group—are inactive, (3) the compound corresponding to B but with  $\text{NH}_2$  groups in positions 3 and X (instead of  $\text{NH COCH}_3$  groups) is inactive (dose 0.4 mg).

It is important that *T. brucei* and *T. congolense* are both influenced by compound A to a practically equal extent, since the latter trypanosome is either not affected by most trypanocidal agents which act on *T. brucei*, or else responds only to the largest doses, e.g. of a substance such as Bayer 205 which cures *T. brucei* infections in a small fraction of the tolerated dose. Both compounds (A and B) act on a drug-fast strain of *T. brucei* which is completely resistant to the largest tolerated doses of arsacetin.

These phenanthridinium compounds constitute a new type of trypanocidal agent.

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616—008.843.5—008.7 (*Bacteroides*)

#### A NOTE ON THE PREDOMINANCE OF THE GENUS *BACTEROIDES* IN HUMAN STOOLS.

S. S. MISRA

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Eggerth and Gagnon (1933) found that in 91 per cent of a series of human adult stools there was a predominance of obligatory anaerobes which they classified, relying chiefly on morphological and fermentative reactions, as members of the *Bacteroides* group. Weiss and Rettger (1937), examining the stools of 40 adults, isolated 87 anaerobic strains which they divided into Gram-positive *Bacteroides* and Gram-negative *Bacteroides*, subdividing the latter by serological methods into 4 main groups. In these stools also the anaerobes predominated over the aerobes. This note reports a quantitative comparison of the numbers of obligate anaerobes and aerobes in 10 adult human stools.

**Method** About 0.5 c.c. of stool was emulsified in 15 c.c. of 0.85 per cent sterile saline and centrifuged for 5 minutes at 1000 r.p.m. to deposit the coarser particles. Tenfold dilutions of the emulsion were made in nutrient broth, using 50 dropping pipettes (Donald, 1916), and 0.02 c.c. of each dilution was dropped on to numbered sectors of six well dried blood agar plates. After the absorption of the drops, three plates were incubated aerobically and three anaerobically at 37° C. The mean number of colonies developing in drop areas that yielded discrete colonies was determined from counts of the three plates made after 24 and 72 hours' incubation. Representative aerobic and anaerobic colonies were examined morphologically and biochemically.

The aerobes were mostly coliform in type and were tested in lactose, sucrose and salicin peptone water and Kosor's citrate medium. With the exception of one strain, a late lactose fermenting paracolon bacillus, all the coliform organisms belonged to the *coli aerogenes* group. Occasionally a few colonies of *Str. fecalis* appeared from the higher dilutions of the faecal emulsion.

Five representative anaerobic colonies from each of the ten specimens were tested by two further 3 day subcultures on blood agar under aerobic and anaerobic conditions. Two of the fifty were facultative aerobes, the remainder proved to be strict obligate anaerobes.

Two or three strains from each specimen were purified by two replatings. Of 26 strains so prepared, 24 were Gram negative, 20 gave, on 3 days' incubation on blood agar, smooth, convex, and entire edged translucent colonies 0.2-0.5 mm in diameter, 4 gave semi-transparent, granular, effuse colonies 0.4-0.8 mm in diameter. The two Gram positive strains gave smooth, entire edged opaque colonies 0.4-0.8 mm in diameter.

All the bacilli were 0.6-0.75  $\mu$  in width, and varied in modal length from 0.8 to 3  $\mu$ . The majority of strains were highly pleomorphic, there was no relation between cell type and colony type.

Table I summarises the biochemical reactions of 18 of the Gram negative strains. All were non-haemolytic, failed to reduce nitrates and, with the exception of 1 C, failed to liquefy gelatin. Lead acetate agar was blackened by 2 strains.

Growth was feeble or absent in heart extract broth, good in 5 per cent blood or serum broth. There was no growth in peptone water but carbohydrate were fermented with the production of acid in peptone water containing 5 per cent serum. No morphological evidence of spore formation was noted and 3 day cultures of all strains were killed after 30 minutes at 80° C.

The Gram negative obligate anaerobes appeared to correspond with Weiss and Rottger's groups I and II although in the absence of fuller biochemical tests and of pathogenicity and serological tests identity cannot be established. The chief difference lies in the greater variability of these strains in their action on litmus milk and lead acetate agar.

Table II shows the count results in the series of 10 stools. The findings of Eggorth and Gagnon and of Weiss and Rottger are confirmed. Spore forming anaerobes were never present in large enough numbers to appear in the higher dilutions of faecal emulsion and the predominating anaerobe may be assumed to be of the *Bacteroides* type. One stool only showed a slight predominance of aerobes. In the others the number of *Bacteroides* like organisms was from 3 to 170 times as great as that of the coliform organisms.

TABLE I

*Biochemical reactions of gram-negative anaerobes isolated from human faeces*

Strain	Glucose	Maltose	Manitol	Lactose	Sucrose	Sallein	Litmus milk	Remarks
1B	A	O	O	A	O	O	A ALK	7 C granular colony
7B, 9B	A	O	O	O	O	A	ALK	
2B, 7C, 8B,	A	A	O	A	O	A	A -ALK	
9A, 10A							or ALK	
4A, 5B, 6A,	A	A	O	A	A	O	AC	
6B, 8A, 10B								Gelatin liquefied, H <sub>2</sub> S+, granular colony
1C	A	A	O	A	A	A	AC	
3A	A	A	O	A	A	A	NC	H <sub>2</sub> S+ Granular colony
4B	A	A	O	A	A	A	ALK	
5C	A	A	A	A	A	A	AC	

N = neutral  
ALK = alkaline

A = acid  
C = clot

TABLE II.

*Aerobic and anaerobic counts in ten specimens of faeces*

Serial no. of faeces	Three-plate counts with standard deviations (millions of viable organisms per c.c. of faecal emulsion)		Approximate ratio of anaerobic to aerobic count
	Aerobic	Anaerobic	
1	4.5 ± 0.50	58.5 ± 10.40	13.0
2	80.0 ± 13.25	26.5 ± 7.65	0.3
3	15.2 ± 1.77	45.0 ± 13.25	3.0
4	No growth (very dry stool)	1.9 ± 0.38	
5	20.0 ± 5.00	1865.0 ± 340.50	93.3
6	2.9 ± 0.30	485.0 ± 76.50	170.0
7	13.4 ± 3.62	139.5 ± 7.65	10.4
8	4.7 ± 0.30	55.0 ± 13.25	11.8
9	1.0 ± 0.28	9.5 ± 1.91	9.5
10	0.4 ± 0.13	5.7 ± 1.16	16.1

*Summary*

Of a series of 10 stools examined, 9 showed marked preponderance of the obligate anaerobes over the obligate and facultative aerobes. The obligate anaerobes appear to be members of the *Bacteroides* group; a further and more extensive study of this interesting genus in faeces might determine what role, if any, its members play in normal and abnormal intestinal conditions.

My thanks are due to Dr A. A. Miles for his interest and advice.

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576 809 4 576 851 4 (*Bact actinomycetem comitans*)*BACTERIUM ACTINOMYCETEM COMITANS* A DESCRIPTION OF TWO STRAINS

N E GOLDSWORTHY

*From the Department of Bacteriology in the University of Sydney, Australia*

Although the literature on actinomycosis is now large, certain fundamental questions concerning the natural reservoirs of the parasite, the immediate source of infection and the mechanism of the pathological processes remain unanswered. The very frequent presence in actinomycotic lesions of organisms other than the actinomyces itself has been commented upon by several authors (Klinger, 1912, Colebrook, 1920, Axhausen, 1936). One of the best known of these organisms is the *Bacterium actinomycetem comitans* (*Actinobacillus actinomycetem comitans*). Colebrook found it in 24 out of 30 lesions and cultivated it from 10. This suggests that the bacterium may be of pathological importance, and a small preliminary series of experiments carried out in this laboratory would seem to support this suggestion. On this account, and because there are very few recorded studies of the organism, the following observations are reported.

*Source of the organisms.* Strain F was recovered from pus from a closed abscess in a cervical lymphatic gland (human subject). Such closed lesions are uncommon and offer rare opportunities for easy recovery of the organisms involved. Bayne Jones (1925) worked with material from a similar situation. Strain R was isolated from a supposedly pure culture of *Actinomyces bovis* (Wolff and Israel) which had been obtained two or three years previously. The patient had actinomycosis of the lung, with abscesses in the forearm and sternum. The culture was grown from pus from the forearm.

*Isolation in pure culture.* Both strains were encountered under the most favourable conditions possible and were readily isolated by taking advantage of the different oxygen requirements of the bacterium and the actinomyces. Broth tubes were inoculated and incubated in an inclined position in order to allow the freest possible access of the air. After 48 hours small colonies were visible on the walls of the tube, and by subculture and plating the bacterium was readily isolated in pure culture.

*Morphology.* In films of pus stained by Gram's method, strain F appeared exactly as described and figured by Colebrook. The Gram positive mycelium of the actinomyces was embedded in a more or less homogeneous Gram negative mass which, at the periphery, resolved itself into small coccobacillary elements. These closely resembled *H. influenzae* and *Br. melitensis* in size, shape and staining properties. Since the bacterium does not stain deeply it is desirable to use dilute carbol fuchsin rather than neutral red as a counterstain in Gram's method. Films from pure cultures in broth showed similar very small, Gram negative bacilli with a strong tendency to form dense clumps. The morphology of two organisms was identical and both strains were non motile.

*Cultural characters.* In broth, the appearance was exactly that described by other writers—small discrete colonies clinging to the wall of the tube, dislodged only with difficulty and not easy to emulsify. On repeated subcultivation in broth the organism lost this tendency and grew diffusely (Colebrook, 1920, Lieske, 1921).

On solid media such as serum agar good growth occurred in 48 hours. On this medium, which seemed to be the most satisfactory one for both strains, the colonies were semi-transparent and small—not more than 1 mm

in diameter and generally less, the surface was smooth and shiny, the shape domed and the edge entire. At times strain R (from the old mixed culture) produced mainly "rough" colonies. Both strains grew fairly well on blood agar (horse and sheep).

Maintenance of cultures is not difficult. In mixed culture strain F has been maintained for one year in cooked meat medium, with monthly transfers and continuous incubation at  $37^{\circ}\text{C}$ , strain R for 3 years. In pure culture both strains have been maintained for a period exceeding one year by weekly transfers on serum agar slopes kept at  $37^{\circ}\text{C}$ .

*Oxygen requirements.* The bacterium is essentially an aerobe and grows freely though somewhat slowly on the surface of solid media. It is also a facultative anaerobe, as shown by its maintenance in mixed culture with *A. bovis* in cooked meat medium under a seal of liquid paraffin.

*Fermentation reactions.* Tests in ordinary peptone water even when enriched with 10 per cent horse serum were unsatisfactory, although growth in the serum medium was good. Slopes of a solid medium consisting of nutrient agar, Andrade's indicator and 1 per cent of the desired carbohydrate gave constant results, though slight fluctuations in the apparent degree of acidity were occasionally seen. Both strains fermented glucose, mannitol, maltose and starch but not lactose, sucrose, dulcitol, inulin, salicin or glycerol. No gas was produced in liquid media even when very large inocula were used. Colebrook found his strain to ferment glucose, lactose, sucrose, mannitol dulcitol and dextrin. Dextrin was not used in my experiments. Acid production was slow and was sometimes delayed until the fifth day. In some cultures the acidity was less marked on the 13th than on the 5th day.

*Hemolysis.* On blood agar plates the bacterium grew moderately well but no hemolysis was observed after five days' incubation. There was, however, a distinct tendency towards greenish discoloration beneath the growth, more evident with strain F.

*Liquefaction of gelatin.* Slab cultures incubated for 14 days at approximately  $20^{\circ}\text{C}$  showed only very slight growth and that at the surface. Cultures incubated in an inclined position at  $37^{\circ}\text{C}$  for a similar period showed better growth. No liquefaction occurred in either case.

#### DISCUSSION.

It is clear that the organisms described above are very similar to those studied by Klinger and by Colebrook, and designated by them *Bacterium actinomycetorum constanti*. The only differences which emerge from these observations relate to the fermentation reactions. So few strains have been studied that the significance of these differences must be uncertain. It is worth noting, however, that the strains F and R, isolated from lesions at very different times, one fresh from the host when tested, the other maintained for 2 years in the laboratory before being tested, had precisely the same reactions. Furthermore, these reactions were constant when the tests were made in several different kinds of basal media—agar, broth and peptone water. The difference in the fermentative abilities of Colebrook's organism may merely indicate the existence of distinct types of the organism, as suggested by Colebrook himself, although he obtained no definite evidence of this.

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576 809 737 616—006 42

THE FOURTH COMPONENT OF COMPLEMENT EXPERIMENTS  
WITH A TRANSPLANTABLE SARCOMA OF THE GUINEA PIG

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The circumstance that a guinea pig tumour is being propagated at present in these laboratories afforded an opportunity of making observations on the influence of malignant growth on serum complement. Attention was directed particularly to the haemolytic titre of complement in such animals and to the possibility of change in the fourth component (Gordon, Whitehead and Wormald, 1926). The tumour in question, the Doels and Biltris cory sarcoma, is one of those originally produced by the introduction of radio active sources into various parts of the body and described by Biltris (1933) as a "sarcoma fuso cellulare intra abdominali qui a puero misca serie". The tumour was started in Leeds from an animal kindly supplied by the laboratories of the Imperial Cancer Research Fund. It was grafted in the usual manner into the right flank.

Seventeen animals in all were used, and the distribution of growth in them was as follows

- (a) Well developed primary tumour at site of graft only (9 guinea pigs, in 5 of which ulceration of the overlying skin had occurred)
- (b) Primary tumour with few metastases (2 guinea pigs)
- (c) Primary tumour with abundant metastases and consequent blood stained ascites (5 guinea pigs)
- (d) Graft, after a period of growth, subsequently regressed (1 guinea pig)

The time from grafting to the killing of the animal for the testing of serum varied from 47 to 68 days with an average of 60 days. This approximates closely to the usual survival period after tumour grafting.

In the tests for complement activity 0.05, 0.1, 0.2 and 0.3 c.c. quantities of serum diluted 1/12 in normal saline were added to 0.5 c.c. of a 4 per cent suspension of sensitised ox corpuscles and incubated at 37° C for 2 hours. Usually 0.05 c.c. gave partial haemolysis and 0.1 c.c. or more complete haemolysis. Within the limitations of our present methods of estimating complement activity the results showed substantial agreement with those obtained with normal guinea pigs, so that there was no appreciable diminution of complement in the tumour bearing animals.

The fourth component was studied by mixing two 3 c.c. amounts of serum obtained from each normal or tumour bearing guinea pig with 0.2 c.c. and 0.3 c.c. respectively of  $NH_4OH$  after incubation for 1½ hours at 37° C and adjustment to pH 7.5 with  $NH_4Cl$ , 0.1 c.c. and 0.2 c.c. amounts from each of these mixtures were added to 0.5 c.c. of a 4 per cent suspension of sensitised ox corpuscles and incubated for 2 hours at 37° C. No haemolysis occurred. These results indicate that the fourth component was inactivated by the ammonia and they were obtained with all sera examined.

A further experiment was undertaken to determine the heat lability and amount of the fourth component in the sera of the tumour bearing guinea pigs as compared with the normal animals. If normal guinea pig serum is heated at 57° C for half an hour, its fourth component is unaffected and if the heated serum is added to ammonia treated serum it restores the

complement activity The sera from tumour-bearing and normal guinea-pigs were heated at 55° C and 0.1 c.c. of each was added to 0.1 c.c. of ammoniated and neutralised sera obtained from both normal and tumour-bearing animals In 16 out of the 17 guinea-pigs with tumours, the fourth component was unaffected by heat, i.e. the addition of their heated serum to ammonia-treated serum led to complete hæmolysis The exceptional serum was obtained from a guinea-pig bearing an ulcerated primary growth with no metastases In the absence of any exact knowledge of the nature of the fourth component it is difficult to explain this one result but we do not feel satisfied that it was due to an error in technique The other 16 sera, after being heated at 55° C for half-an-hour, were diluted 1:3 with normal saline and amounts of 0.05, 0.1, 0.2, and 0.3 c.c. were added to 0.1 c.c. of ammoniated serum from normal and tumour-bearing animals, when sensitised cells were added to these it was found that the reactivating capacity of the heated sera was quantitatively similar in both groups of animals.

One experiment was done on a rabbit which had been inoculated intratesticularly with the Brown-Pearce (1923) carcinoma and showed at autopsy extensive visceral metastases The serum of this rabbit as compared with that of several normal rabbits showed no quantitative loss of complement, while the fourth component could be inactivated by ammonia and its heat stability and quantitative activity were not affected by the presence of the tumour

#### Summary.

An examination of the serum complement in seventeen guinea-pigs and one rabbit bearing transplanted tumours, in which especial attention was devoted to the fourth component, has failed to reveal any abnormality except in one guinea-pig.

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AND WORMALL, A.

## OBITUARY NOTICE OF DECEASED MEMBER

### William McIntosh Cumming.

1899-1937

DR WILLIAM MELROSE CUMMING, late lecturer in bacteriology in the University of St Andrews, died suddenly on 15th August 1937.

Born at Dundee on 17th November 1899, Cumming received his early education at the Morgan Academy in that city and his professional training at the School of Medicine of the University of St Andrews. Graduating in 1922, he was immediately appointed assistant to the professor of bacteriology, a post in which he soon displayed those qualities of aptitude, initiative and energy which continued to mark all his subsequent work. From the earliest days Cumming showed a strong bias towards the study of tuberculosis and in 1926 he received the doctorate of philosophy for a thesis on the glycerol reaction curve of cultures of *Mycobacterium tuberculosis*. This and much of his later work was performed with the assistance of grants from the Medical Research Council, a form of encouragement and recognition of which he was very appreciative and of which he often spoke.

In 1924 he forsook the work of the laboratory for that of the wards and during the next two years received an intensive training in the clinical study of tuberculosis under the tutelage of Dr W. T. Munro of Glenlondond Sanatorium. In 1926 he was appointed medical superintendent of the Bradford City Sanatorium at Grassington, Yorkshire, where, in addition to his routine clinical and administrative duties, he was soon busily engaged on further bacteriological studies in the little laboratory which he had had equipped. His enthusiasm as a laboratory worker was only equalled by his aptitude as a clinician and in his hospital on the wind-swept hillside looking across Wharfedale to the lovely Fells of Thorpe and Burnsall, he was able to find full scope for both activities. During the period 1924-35 he published numerous papers which received perhaps less publicity than they merited because of their appearing in specialist journals. Most of these dealt with tuberculosis, but his interests remained wide, as is shown by the fact that his thesis for the doctorate of medicine, which he received in 1927, and for which he was awarded a Rutherford medal, dealt with the subject of the streptococci. Also he recorded most fully a case of extreme



hypersensitiveness to cow's milk protein, the case being that of his own child

The death of his wife about three-and-a-half years ago deeply affected him and ultimately resulted in a breakdown during 1935. With the establishment of convalescence he returned to laboratory work with renewed ardour, and in November 1935 made what was to be his final contribution to the literature of his subject—"The type of the causal organism in 1,502 recent English and 320 recent Irish cases of pulmonary tuberculosis" (*Tubercle*, 1935-36, xvii 67). It says much for his enthusiasm that, although not yet fully restored to health, he undertook and carried to completion so laborious a piece of work.

While the furtherance of his speciality was the main factor in his life, Cumming had many other interests. A keen fisher, he loved the lochs and streams of western Scotland, he was also quite handy with a sporting gun and he had a passion for dogs. His real pastime, however, was of a gentler nature—the study of bird life. Indeed to know Cumming it was necessary to understand his enthusiasm for this, one of the most arduous though perhaps the gentlest of all hobbies. His death is a sad loss to those of us who knew him well and we must sympathise with his little son and daughter and with his devoted parents in their bereavement.

W J T  
M J S

## BOOKS RECEIVED

### A textbook of plant virus diseases

By KENNETH M SMITH London J & A Churchill 1937 Pp x and 615 102 figs (1 in colour) 21s

Those working in agriculture, horticulture and virus research should find this book extremely useful, bringing together as it does, under one cover, a vast amount of information upon the various virus infections to which plants are liable.

In the commonly accepted sense of the word it is a "handbook" rather than a "textbook." The student desirous of knowing more about actual technique and methods and the theories underlying virus research is referred to Dr Smith's book in the "Recent Advances" series (reviewed in this *Journal* 1935, vii 573). In form the present book has been deliberately constructed so as to permit the field worker to make a rapid preliminary diagnosis and to follow this up by means of cross references to the more detailed information on symptoms, transmission and control contained in the body of the book. Ultimately, by means of the list of references appended to each chapter, the original sources may be located.

This system of classification of the viruses has been drastically and carefully reorganised and an easily remembered nomenclature has been evolved which is based on the chief host plant of each virus. This is a considerable step forward and not only introduces a simplification of the present rather easily confused nomenclature but also has the advantage of permitting great adaptability and easy inclusion of new viruses as soon as they are discovered. In the case of every virus, the old names and synonyms are given in full.

In the first seven chapters, which form the bulk of the book the virus diseases are dealt with according to their most important host plant. The symptoms of infection are given clearly and fully together with the geographical distribution, range transmission factors and methods of control. In the case of transmission by insects, a later chapter deals very fully with the means of identification habits, etc., of these insect vectors.

### Muir and Ritchie's Manual of bacteriology

Revised by C H BROWNING and T J MACKEN Tenth edition London Humphrey Milford Oxford University Press 1937 Pp x and 996 26 figs in colour on six plates and 212 text figs 20s

The appearance of this, the tenth, edition of Muir and Ritchie just forty years after the first calls for congratulation rather than comment. This particular edition is noteworthy for the fact that for the first time Sir Robert Muir has taken no share in the authorship.

The book remains what it always has been, a solid compilation packed full of facts accurate and well up to date. It is about 170 pages longer than its predecessor and there has been much rewriting and a little rearrangement. One could have wished this might have gone

further in the first edition under the new regime. This book has always been difficult to read and has perhaps been more popular for reference than as an introductory textbook. One reader at any rate has thought this was due to the arrangement of the earlier chapters and the interpolation of chapters II to IV on methods between chapters on the general aspects of the subject. This arrangement has seemed to confuse and distract, and could have been avoided by placing them right at the end of the volume. At the present rate of increase in size it will probably be necessary in the future to consider whether these chapters and those on protozoa and fungi will be able to remain, though their disappearance would alter seriously the character and value of the book. The format is unchanged, but the printing is extraordinarily uneven on many of the earlier pages and half of p. 269 in the copy sent for review is very nearly illegible.

### **Einführung in die Hygiene und Seuchenlehre**

By HANS ZIESS and ERNST ROEDERSWANDT. Second edition. Stuttgart 1937. Pp. viii and 282. 1 text fig. RM 7.60.

The first edition of this book, published in 1936, has already been reviewed in this *Journal* (1936, xliii, 230). The second edition has been considerably extended. To the first section of the book, dealing with hygiene there are added chapters on industrial disease and its control, on disinfection (*Desinfektion*) and destruction of various parasite carriers of disease or destroyers of foodstuffs, etc. (*Entwesung*), and lastly on racial hygiene. In the last, the recent German laws on the subject of marriage are discussed at length in the light of heredity, national honour, morality, etc.

A short general discussion of virus disease has been added to the second section of the book, which deals with infectious disease.

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

7th and 8th JANUARY 1938

The fifty sixth meeting was held in the Bland Sutton Institute of Pathology the Middlesex Hospital, London, on 7th and 8th January 1938

## Communications and demonstrations

Those marked \* are abstracted below

- \*C S HALPERIN Some leading principles in the pathology of the internal ear and of the labyrinth capsule
- L R WOODHOUSE and PIERCE Classification of the melanomata
- H A MAGNUS The gastric lesion in pernicious anaemia
- T B DAVIE Massive fat replacement of the pancreas
- G R CAMFISON and G S W DE SARAY Nembutal and liver damage
- C V HARRISON and P WOOD The study of the coronary arteries by barium injection and radiography
- I H BIGNARD and G ALFANDER Experimental diabetes insipidus
- P A GORIN Antigene basis of tumour transplantation
- T LUNDSFV A simple agglutination test of resistance to tumour implantation
- W CHAMBER and E S HENNING The prevention of spontaneous cancer of the mamma in the mouse
- J W OUN The histological course of oligoma in mouse skin treated with carcinogenic hydrocarbons
- C H ANDREWS and C G AHLSTRÖM The action of tar in some virus infections
- J CZERNESKY The influence of X radiation on the growth of heterologous tumour grafts
- F R SELBIE Tumours in rats and mice following the injection of thorotrast
- P BROWNING The action of synthalin in experimental trypano-somiasis
- E J KING and M McGEORGE The dissolution and excretion of silica
- P HANTLEY Anaphylaxis in guinea pigs to diphtheria toxin
- JRAN ORR EWINN The relative susceptibility to phagocytosis of *gravis* and *mitis* types of *C. diphtheriae*
- C G POPE and MANNART M HALLIV Nitrogen content of floccules produced from refined diphtheria antitoxin
- C L OAKLEY and C G POPE Particle size of concentrated and refined diphtheria antitoxin as determined by ultrafiltration analysis
- EMMA KLEINBERGER Further experiments (serological and infective) with the filterable organisms recovered from rat pulmonary lesions and other sources
- J CRICKSHANK An organism exhibiting mobile colonies
- A W DOWNIE Observations on a virus obtained from pustular lesions on the hands of a cowman
- C E VAN ROOYEN (1) Micromanipulation and microdissection of the molluscan contagious inclusion body (2) Specific complement fixation tests with Shope's fibroma virus and their relationship to virus neutralising antibodies
- VIRGIL ROBERTSON Some interactions between protozoa and immune bodies
- F S DUTHIE A case of carcinoma of the stomach with unusual metastases
- C S HALPERIN (1) Histological technique of the temporal bone (2) Histological preparations illustrating the normal and pathological anatomy of the temporal bone
- T B DAVIE (1) Two cases of syphilitic aortitis in children (2) Undiagnosed intrathoracic chloromatous tumours

- DORIS M. STONE and DOROTHY WOODMAN Polycythemia terminating in leuco-erythroblastic anemia
- L. COLEBROOK, W. R. MAXTED and R. G. WILLCOCKS A simple agitator to work in a water bath
- \*A. J. RHODES The effect of intracerebral passage on the virus of infectious myxomatosis of rabbits
- A. J. RHODES and C. E. VAN ROOYEN An infective disease of uncertain aetiology in a stock of laboratory rats
- C. J. POLSON \*(1) Bronchial carcinoma at the apex of a lung (2) "Cryptic" bronchial carcinoma
- G. P. GLADSTONE Staphylococcal  $\alpha$ -haemolysin production in a chemically defined medium
- W. E. CARNEGIE DICKSON and F. W. WILLWAY Spinal meningiomata
- W. E. CARNEGIE DICKSON, J. PLESCH and A. WEBB-JOHNSON So-called "hypernephroma" of the kidney
- L. E. GLYNN, M. L. ROSENTHAL and E. G. WHITE Mesenteric chyladenectasis with steatorrhea in man and two cases in cats
- R. DORRIS and G. S. W. DE SARAN A case of haemorrhagic encephalitis associated with acute rheumatism
- DOROTHY M. VAUX Lymphadenoid goitre
- J. R. M. INNES Testicular tumours in animals
- JOAN M. ROSS Cutaneous carcinoma following irradiation
- E. G. WHITE Pituitary tumour in a dog

## Abstracts.

616. 281

## SOME LEADING PRINCIPLES IN THE PATHOLOGY OF THE INTERNAL EAR AND OF THE LABYRINTH CAPSULE

C. S. HALLPIKE

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Following birth, the increase in size of the cavities of the mammalian internal ear is exceedingly small. Associated with this, there is a marked absence in the deeper layers of the labyrinth capsule of the normal processes of resorption and re-formation to which other parts of the skeleton are subject. This anomalous physiological constitution of the deeper layers of the labyrinth capsule is of importance in considering the course pursued in the temporal bone by Paget's disease, osteitis fibrosa, and other generalised disorders of the skeleton. The changes in these diseases are mainly confined to the outer or periosteal layer of the labyrinth capsule, the deeper layers being involved only in the more advanced stages of the disease.

In otosclerosis, a disease peculiar to the labyrinth capsule, certain anomalies in the histological changes in the marrow spaces have long presented difficulties in interpretation. On the whole, the nature of these changes has been considered to contradict the possibility that they represent an inflammatory reaction to a chronic infective process. In assessing the changes in question, the necessity should be emphasised of attaching due weight to the possibility that their atypical nature may be attributable, in part at least, to the peculiarities in physiological constitution of the tissue in which they arise, namely the labyrinth capsule.

The neural elements of the mammalian internal ear exhibit certain anomalies in their relation to section of the trunk of the 8th nerve. Consequently upon this operation, the peripheral vestibular neurone with Scarpa's ganglion is preserved following transection of the nerve chord, in accordance with the Wallerian law. The peripheral cochlear neurone with

its spiral ganglion fails to behave in accordance with this law and exhibits severe and permanent degenerative changes. It is considered likely that this may be attributable to some unusual degree of vulnerability of the peripheral cochlear neurone to section of its centripetal axons as also to other types of injury.

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## THE EFFECT OF INTRACEREBRAL PASSAGE ON THE VIRUS OF INFECTIOUS MYXOMATOSIS OF RABBITS

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Hurst (1937) was the first to demonstrate the effect of intracerebral passage on the virus of infectious myxomatosis of rabbits. In his hands the virus altered in its characters during 23 consecutive passages. The histological picture lost its typically myxomatous appearance after about 10 passages and correspondingly the virus became less virulent on intradermal injection, a considerable number of animals actually recovering from the infection. I have had no difficulty in repeating this work and in detecting the change in histological characters. In my hands, however, the virus has remained remarkably virulent despite 28 serial passages to date.

### *Methods*

The strain of virus, which had already been used for a series of experiments in this laboratory (van Rooyen, 1937, van Rooyen and Rhodes, 1937 38), was kindly supplied by Dr Hurst nearly 2 years ago. It had previously been obtained from America. In this laboratory it has been maintained by occasional testicular passage and has invariably proved fatal to rabbits on intracerebral, intradermal or intratesticular injection.

The first rabbit of the series was injected intracerebrally with 0.2 c.c. of a 1:5 saline suspension of desiccated testis. The animal was killed on the 6th day when moribund from generalised myxomatosis. The brain was removed, tested for sterility, and 0.2 c.c. of a 1:5 saline suspension injected intracerebrally into the second animal. This procedure has been carried out 28 times. At every 4th passage brains were examined histologically and rabbits injected intradermally with 0.2 c.c. of brain suspension. The characteristic papules produced were considerably smaller and less purple with the later than with the earlier injections. The papules were excised, usually on the death of the animal, and examined histologically.

### *Results*

The results of intracerebral injection have been very regular. In almost every one of the 28 rabbits "running" at the eyes began on the 5th day, on the 6th day the animal was acutely ill and on the 7th moribund. The majority of animals were killed when in *extremis* on the 7th day but a certain number were allowed to die from the infection on the 7th or 8th day. No suggestion of any change of virulence has been afforded, therefore, by this route of injection.

Nor with animals injected intradermally has any evidence of permanent loss of virulence been obtained. Both 20th and 24th brains caused death from generalised myxomatosis in 10 days. Intratesticular injection with the 24th brain also produced a fatal result.

As up to the 24th passage only one animal had been injected intradermally whenever the virus was so tested (i.e. at every 4th passage), it was

decided to inject 4 animals with the 28th brain in the hope that at least one might recover. No such result was achieved, all the animals dying from the infection on or before the 10th day. It is evident therefore that in this experiment the "neurovirus" is still unaltered in virulence, a finding which contrasts with that of Hurst. Ledingham (1937) alludes to the unexpected virulence of "neurovirus". Histological appearances, however, have afforded some evidence of modification in the action of the virus.

#### *Histology of the cerebral lesion*

The 1st, 4th and 8th passage brains showed a characteristically myxomatous meningeal reaction. Many typical myxoma cells were found, particularly in the adventitial sheaths of the meningeal vessels. The cellular infiltration was principally eosinophilic in nature. The 16th brain showed a marked diminution in the numbers of myxoma cells although the eosinophil infiltration was unaltered.

The 20th, 24th and 28th brains showed complete loss of the typically myxomatous reaction, no myxoma cells were present and there was a scanty cellular infiltration of mononuclears with only a few eosinophils.

#### *Histology of the skin papules*

The skin papule produced by intradermal injection of unmodified virus presented a typically myxomatous appearance. Numerous myxoma cells were seen. The endothelial proliferation of the capillaries in the deeper layers of the dermis, as originally described by Hurst, was present. The epithelium showed considerable vacuolation and numerous Rivers' inclusions. Precisely similar appearances were produced by injection of 4th and 12th passage brains. The 16th brain, however, showed only a few myxoma cells and only slight evidence of endothelial proliferation although vacuolation and Rivers' bodies were still present in the epithelium.

The 20th, 24th and 28th brains showed complete absence of myxoma cells, endothelial proliferation and vacuolation. Rivers' inclusions were scanty.

#### *Conclusions.*

- 1 Infectious myxoma has been passed intracerebrally 28 times through rabbits and a series of histological examinations made.
- 2 In both brain and skin lesions the histological picture lost its specifically myxomatous appearance in the 20th and subsequent passages.
- 3 No alteration in virulence was associated with these changes.

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#### BRONCHIAL CARCINOMA AT THE APEX OF A LUNG

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The opinion of the majority (Jacox, 1934, Steiner and Francis, 1934, Fried, 1934; Marcl and Crawford, 1936) favours the view that tumours situated at the apex of a lung are probably derived from the epithelium

of a bronchus or bronchiole Pancoast (1924, 1932) suggested that these tumours were a distinct group and termed them superior pulmonary sulcus tumours. In his view and in that of Henderson (1930) most were endotheliomata of the pleura. Pancoast thought that these tumours had characteristic clinical and radiological appearances but the findings in the present case had little in common with the criteria of Pancoast.

### *Clinical and radiological findings*

A man aged 28, a fat refiner, was admitted to St James's Hospital, Leeds, on 24th November 1932. For eighteen months he had had a cough which was at no time severe. During the past six weeks it had become worse but was alleviated by medicine. A month ago he had pain between the shoulder blades, it had never made him cry out. A week later the pain moved into his left shoulder and he likened it to "rheumatism". He sweated profusely by day and night. There was no haemoptysis nor any known loss of weight. Tubercle bacilli were not found in his sputum. On examining the chest a rounded fullness was palpated at the apex of the left lung. This area was tender on deep pressure and dull to percussion, and breath sounds were absent in it. Wasting of the hand and Horner's syndrome were not observed.

Radiological examination on 16th November by Dr Wall revealed an opacity of uniform density occupying the left apex and extending downwards to the level of the neck of the fifth rib. It was defined by a sharply curved border, convexity downwards. The opacity contained oval shaped areas of lesser density, suggesting cavitation. The mediastinal contents were not obviously displaced, no destruction of the ribs was observed.

The patient had considerable fever of intermittent type until 1st December, after which the temperature was never above  $101^{\circ}\text{F}$ . During the illness, the pulse rate ranged between 110 and 120, and the respiration rate between 26 and 36. Each of three blood examinations (22nd Nov, 3rd and 7th Dec) showed polymorphonuclear leucocytosis, with total white cell counts of 30,000, 48,000 and 45,000 per c mm.

On 24th November the left apex was explored by Mr Callinson. A hard mass was felt in the upper lobe of the left lung and repeated attempts to aspirate it were unsuccessful. The patient went steadily downhill after the operation, though he exhibited the signs of phthisis throughout. He died on 16th January 1933.

### *Autopsy findings*

The body was emaciated and unduly pigmented. A dense mass of rubber like consistence and cream colour was present in the upper third of the thorax. It had extended beneath the left clavicle and it was firmly adherent to the thoracic wall. It was intimately related to but had not invaded the brachial plexus, which was stretched over the summit of the growth. Externally the tumour appeared to sit upon the apex of the left upper lobe and, although intimately bound to the lung, was not apparently within it. The growth was somewhat nodular and of cream colour, its external measurements were 13 cm at the base and 7 cm vertically. Section of the left lung showed that there was a considerable intrapulmonary mass measuring  $10 \times 4.5$  cm. This was irregularly cavitated centrally in an area  $5 \times 2.5$  cm. The cavity contained pulsatious necrotic tumour tissue and fluid of the consistence and appearance of thick cream. The lung elsewhere was the seat of mild compensatory emphysema, as was the right lung, from the surface of which several small bullae projected. Although the bronchi



of the left upper lobe were traced to the furthest practicable limits, namely to branches of 3 mm. diameter or even less, macroscopic origin of the growth from a bronchus was not demonstrated

Secondary deposits of small size were present in the mediastinal lymphatic glands, in a coeliac lymphatic gland and in the right kidney cortex (5 mm diameter) Both suprarenal glands were destroyed by secondary carcinoma The right measured  $5 \times 3 \times 5$  cm, the left  $10 \times 7 \times 5$  cm Except for the osseous system, which was not examined, no other structures, including the brain, contained secondary deposits The heart (7 oz) was the seat of brown atrophy

#### *Microscopical examination*

Twelve blocks of tissue from different areas and representative of the primary growth as a whole, together with portions of both suprarenal glands, lymphatic glands, kidney, liver and spleen, were examined

The primary growth in several blocks, especially from the periphery, was distinctly anaplastic, being composed of large polyhedral mononuclear cells set within thin strands of connective tissue No definite arrangement was observed There was also an abundance of multinucleated cells  $40-70 \mu$  in diameter, with central hyperchromatic nuclei Some of these cells appeared to contain leucocytes, when present in areas widely infiltrated by polymorphs In one area the growth had a glandular appearance Elsewhere the growth had the characters of a much de-differentiated squamous cell carcinoma Various degrees of anaplasia were observed In two preparations from the centre of the intrapulmonary portion of the growth, here mainly composed of necrotic material, small islands of unmistakable squamous-cell carcinoma were present, containing imperfect epithelial pearls Pickle cells were not demonstrated The secondary deposits were all anaplastic, and multinucleated giant cells were plentiful at many points

#### *Commentary*

A clinical diagnosis of pulmonary abscess was most favoured on account of the age of the patient, high fever, a cavitated mass with a well defined lower border at the lung apex, high polymorphonuclear leucocytosis and the absence of tubercle bacilli in the sputum Whilst some of the microscopical specimens might have justified a diagnosis of pleural endothelioma, the epithelial structure of the tumour was established and, although the point of origin was no longer demonstrable, it is probable that the growth originated from a small branch of the bronchial tree Borst has described a pleural endothelioma containing epithelial pearls but Ewing (1928) is dubious about his diagnosis

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## THE EFFECT OF X-RADIATION ON THE BLOOD AND LYMPHOID TISSUE OF TUMOUR-BEARING ANIMALS

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(PLATE XIII)

AN attempt was made in this Institute in 1934 to grow a mouse sarcoma (Mal sarcoma 1) in rats and a number of stock, Wistar and Middlesex rats were grafted with the tumour. In all, 65 rats were inoculated, in 23 of which the sarcoma grew for a short time before retrogressing, but after the 2nd passage the takes were negligible and the experiments were discontinued.

As stated in a previous paper (Mayneord and Parsons, 1937) it had been found that general irradiation of mice under treatment with a chemical compound—sodium 1, 2, 5, 6 dibenzanthracene 9, 10 endo  $\alpha\beta$  succinate (Cook, 1931)—accelerates tumour formation and increases the number of sarcomas induced. It was therefore thought that irradiated rats might be less resistant to inoculation with the Mal sarcoma and the experiments detailed below were undertaken.

Recently Clemmesen (1937) has tested the effect of X-radiation on rats implanted with mouse sarcomas and has shown that, although the grafted tumour eventually retrogresses in the heterologous animal, yet the number of takes is increased, the tumours are of larger size and serial passages can be carried through a longer period than in non irradiated animals. He mentions that a heavy mortality occurred amongst rats irradiated with 500 600 r and a similar mortality was found in the present experiments. The cause of this high death rate was therefore investigated and certain

pathological conditions were found in practically all rats succumbing before the 20th day

#### *X-ray technique.*

The animals were placed in a wooden box partitioned into two sections each measuring  $17.7 \times 13.4 \times 18.5$  cm (inside measurements). The sections could be irradiated in turn, the rats in the section not being irradiated being screened from stray radiation by a lead sheet of 1 mm thickness covering the partition and a similar sheet placed over that portion of the lid of the box.

The X-ray tube was a Philips "Metalix" operated at 150 kv and 4 milliamperes by a constant potential generator. The chrome-iron filter incorporated in the tube was equivalent to 0.3 mm of copper. No additional metal filter was used. The beam was limited by a brass cone 8.5 cm in diameter at the base, 0.35 cm in thickness and 8.0 cm high. The distance of the focus of the tube to a point 1 cm above the bottom of the animal box was measured before an irradiation and was normally about 31.5 cm. The dosage rate was checked with a Siemens dosimeter before the exposure of every batch of animals. It was approximately 30 r/min at this distance measured in air. A correction of 4.5 per cent, determined experimentally, was made to allow for absorption of radiation in the wooden lid of the box, which was 9 mm thick. Under the conditions of the experiment the whole of the body of each animal was irradiated.

#### *Other experimental details*

Black and white stock rats (males) of about 100-130 g weight were employed and were subjected to X-radiation in varying dosage previous to grafting the sarcoma. In all, 35 rats were inoculated (table I), and as controls a further 13 rats received irradiation only. The tumour material was introduced *sub cutem* into the right flank by means of a trochar and cannula, the graft measuring approximately 2 mm. cube.

### RESULTS

Fig. 1 shows the number of takes and the growth of the tumours to the 4th generation. When compared with non-irradiated controls the number of takes was higher and the tumours larger, yet many of the rats irradiated with 300-600 r died from the 5th to the 12th day after inoculation and many surviving this period succumbed before the 20th day. It was noted that the feet were blanched and the animals appeared to suffer from air hunger. Examination of the blood showed a great fall in the number of red cells and in the hæmoglobin content. Animals appearing to suffer from this anæmia were therefore killed when the symptoms developed, and certain features both in the blood and in different organs were found in most of the animals irradiated above 300 r. Rats receiving 50-100 r remained healthy, only slight falls occurring in their red cell counts.

#### *Changes in the blood*

In rats surviving irradiation, the blood showed a slight fall in the total red cell count. In those dying from the effects of

radiation the blood appeared pale and watery. Coagulation seemed to be delayed, though no estimation of this was made, and the resulting clot was of a loose friable nature. The serum was almost colourless.

**Red cell count** A progressive fall in the number of red cells was noticed from the time of irradiation onwards, the final figure

CHART OF GROWTH OF MOUSE SARCOMA MAL1  
IN IRRADIATED RATS

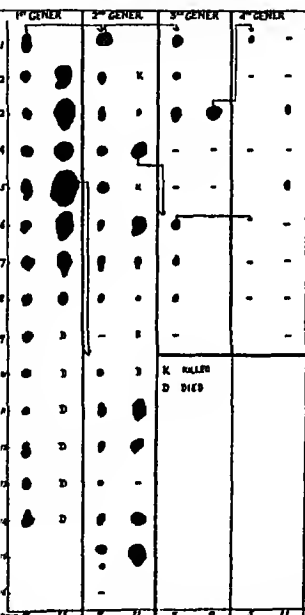


CHART OF GROWTH OF MOUSE SARCOMA MAL1  
IN NON-IRRADIATED RATS

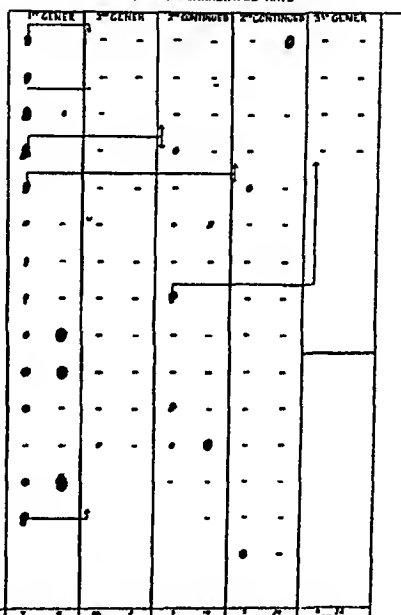


FIG. 1.—Chart showing number of takes and extent of growth of mouse sarcoma in generally irradiated and non irradiated rats

in extreme cases being about a million. (The normal red cell count of 30 untreated rats gave an average of 7,500,000 per c mm.) Films showed extreme poikilocytosis, polychromasia and granular degeneration of the erythrocytes, but almost no normoblasts could be found in any of the films from rats succumbing to the irradiation. In the survivors, normoblasts were present in the blood 3-4 weeks later.

**White cell count** Only a few leucocyte counts were made. The

usual effect of X-radiation—a leucopenia with diminution of the absolute values of the lymphocytes—was observed. In none was a leucocytosis present.

RED BLOOD CELL COUNTS AND COLOUR INDICES OF IRRADIATED RATS  
GRAFTED WITH MOUSE SARCOMA MAL I

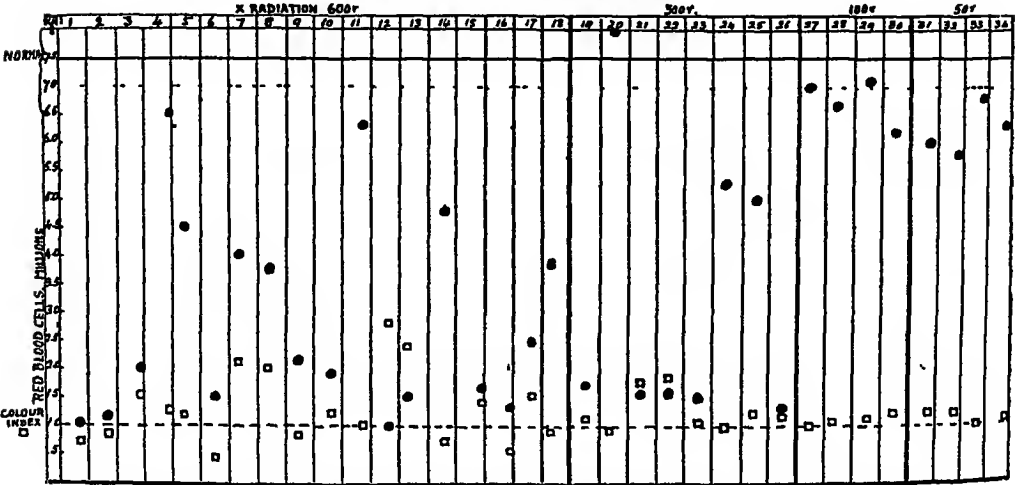


FIG 2—Chart showing terminal counts of red cells and colour index of irradiated rats grafted with mouse sarcoma

IRRADIATED RATS (CONTROLS)  
(NOT GRAFTED WITH MOUSE SARCOMA MAL I)

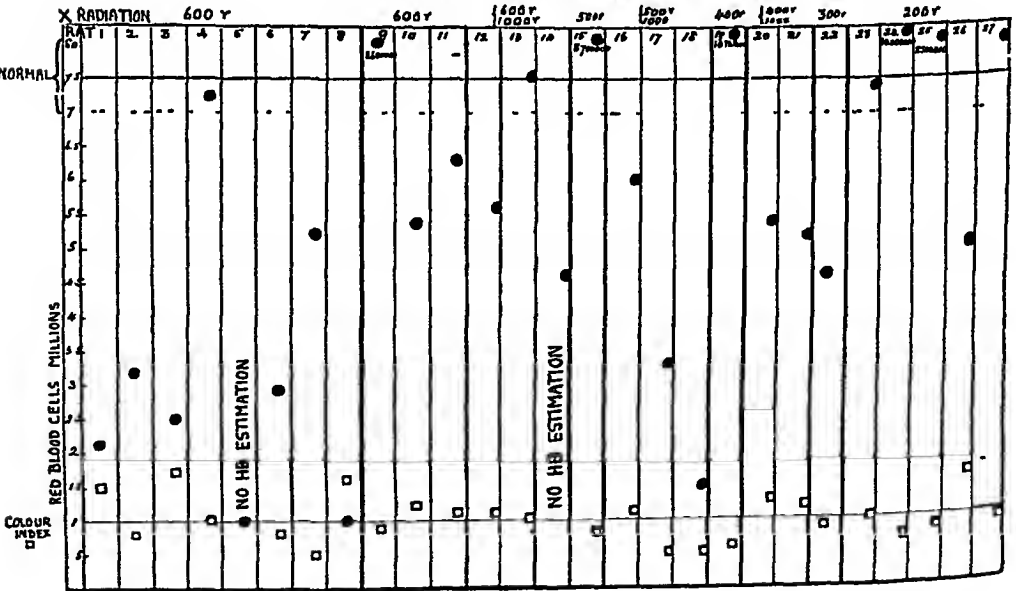


FIG 3—Chart showing terminal counts of red cells and colour index of irradiated rats not grafted with mouse sarcoma

*Hæmoglobin.* Estimations were made with a Dare's hæmoglobinometer, and it was found that a progressive fall occurred

which was not proportional to the decrease in red cells. In the greater number of rats the colour index till just before death was over unity, but in the final stages a profound fall in hæmoglobin took place, and estimates were approximate only, owing to the lack of colour in the blood, which assumed a grey-yellow tint difficult to match against the standard indicator. Figs 2 and 3 give the total number of red cells and the colour index in terminal counts of individual rats, and tables I and II show these data in

TABLE I  
Changes in irradiated rats grafted with *Mal. sarcoma 1*

Rat no	X ray dose	Length of life (days) D, died, K killed	Terminal counts.			Weight of spleen (g)	Liver
			Hb (per cent)	RBC	OI		
1	000 r	K 13	10	1,000,000	0.7		Fatty
2	000 r	K 13	14	1,200,000	0.8		"
3	600 r	K 20	42	2,000,000	1.5	0.3	"
4	600 r	K 20	00	6,500,000	1.3		"
5	000 r	K 7	74	4,500,000	1.2		"
6	000 r	K 10	10	1,520,000	0.4	0.38	Fatty
7	000 r	D 7	59	4,000,000	1.1		"
8	600 r	K 13	79	3,750,000	1.5		"
9	000 r	K 10	24	2,250,000	0.8		Fatty
10	600 r	K 12	32	1,000,000	1.3	0.37	"
11	000 r	K 15	89	6,300,000	1.0		"
12	000 r	K 11	32	1,000,000	2.8	0.15	Fatty
13	600 r	K 7	45	1,000,000	2.4		"
14	000 r	D 14	44	4,800,000	0.7	0.42	"
15	600 r	K 18	31	1,700,000	1.4	0.0	Fatty
16	600 r	K 18	10	1,300,000	0.5	0.20	"
17	600 r	K 15	54	2,400,000	1.0	0.37	"
18	600 r	D 8	47	3,800,000	0.0	0.34	"
19	300 r	D 11					Fatty
20	300 r	K 11	31	1,800,000	1.2		"
21	300 r	K 11	100	8,000,000	0.0		"
22	300 r	K 11	30	1,600,000	1.8		Fatty
23	300 r	K 10	31	1,600,000	1.9		"
24	300 r	K 10	31	1,500,000	1.1		"
25	300 r	K 12	70	5,300,000	1.0	0.84	"
26	300 r	K 12	70	5,000,000	1.2	0.75	"
27	300 r	K 12	23	1,370,000	1.2	0.20	Fatty
28	100 r	K 8	100	7,000,000	1.0	0.67	"
29	100 r	K 0	100	6,000,000	1.1	0.84	"
30	100 r	K 9	100	7,100,000	1.1	0.80	"
31	100 r	K 0	100	0,200,000	1.2	0.07	"
32	50 r	K 15	100	6,000,000	1.2		"
33	50 r	K 15	100	5,800,000	1.2		"
34	50 r	K 15	100	6,800,000	1.0		"
35	50 r	K 15	100	6,300,000	1.1		"

relation to X-ray dose, length of life, percentage of hæmoglobin and changes in liver and spleen. For convenience all data relating to control animals have been included in table II, nos 1-8 and

no 27 being counts from rats included in the first set of 13 controls mentioned above (p 222) Animals found dead and decomposed are not included.

TABLE II.  
*Changes in irradiated non-grafted control rats*

Rat no	X-ray dose	Length of life (days) D, died K, killed	Terminal or latest blood counts			Weight of spleen (g)	Liver
			Hb (per cent)	R B C	C I		
1	600 r	D 10	40	2,100,000	1 4	0 178	Fatty
2	600 r	D 9	33	3,200,000	0 8	0 2	"
3	600 r	K 13	57	2,500,000	1 7	0 4	"
4	600 r	K 13	100	7,200,000	1 0	0 5	"
5	600 r	D 11		1,000,000		0 1	Fatty
6	600 r	K 11	31	2,900,000	0 8	0 23	"
7	600 r	K 11	37	5,200,000	0 5	0 41	"
8	600 r	K 11	23	1,000,000	1 7	0 37	Fatty
9	600 r	K 11	100	8,600,000	0 9	0 24	—
10	600 r	K 12	98	5,400,000	1 3	0 18	—
11	600 r	K 13	100	6,300,000	1 1	0 24	—
12	600 r + 1,000 r	K 25	84	5,600,000	1 1	0 11	—
13	600 r + 1,000 r	K 21	100	7,500,000	1 0	0 98	—
14	600 r + 1,000 r	K 39		4,600,000			—
15	500 r + 1,000 r	K 14	100	8,700,000	0 8	0 21	—
16	500 r + 1,000 r	K 14	88	6,000,000	1 1	0 56	—
17	500 r + 1,000 r	K 26	.				—
18	500 r + 1,000 r	D 26	23	3,300,000	0 5	0 14	Fatty
19	500 r + 1,000 r	D 26	10	1,500,000	0 5	0 17	"
20	500 r	D 20		.			—
21	400 r + 1,000 r	K 17	100	5,400,000	1 3		—
22	400 r + 1,000 r	K 31	88	5,250,000	1 2	0 46	—
23	400 r	K 31	93	10,000,000	0 7	0 36	—
24	400 r	K				0 2	—
25	400 r	Alive	.			.	—
26	400 r	"					—
27	300 r	D 5	55	4,600,000	0 9	0 98	—
28	200 r	Alive 56	100	7,400,000	1 0		—
29	200 r	" 56	100	10,000,000	0 7		—
30	200 r	" 56	100	8,500,000	0 9		—
31	200 r	" 56	100	4,500,000	1 6		—
32	200 r	" 56	100	8,000,000	0 9		—
33	200 r	" 56	.				—

*Thrombocytes* In all films a thrombocytopenia was noticed

*Changes in various organs*

**Liver** Macroscopically the livers of all rats dying of or killed for anaemia were of a friable consistency and more or less brown-yellow in colour. Sections stained with Sudan III showed advanced fatty change, most marked round the centres of the lobules, in some cases only a small area of normal liver tissue was left at the periphery of each lobule.

**Spleen** The spleen was always reduced in size and sometimes extremely small and it was noted that the atrophy was proportional to the fall in the blood count, rats with a moderate degree of anaemia or in the earlier stages of the disease having spleens larger than those dying with red cell counts of one or two million. The

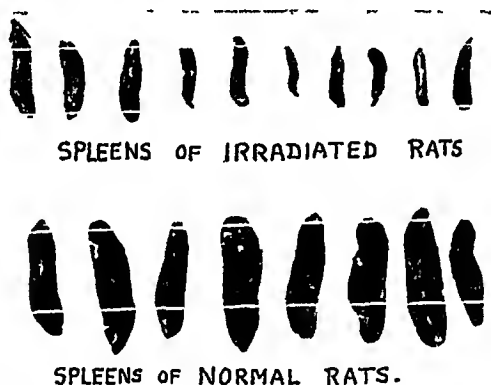


FIG. 4.—Spleens of rats (100–130 g) irradiated 600 r, average weight of 16 = 0.35 g.  
Spleens of normal rats (100–130 g), average weight of 8 = 1.2 g.  $\times \frac{1}{2}$

weight of the spleen in 8 normal rats averaged 1.2 g, the average weight in irradiated rats (fig. 4) was 0.35 g. Microscopically, the splenic tissue showed a decrease in lymphoid elements. The reticulo-endothelial cells gave a marked prussian blue reaction, an absence of giant cells was noted.

**Mesenteric glands** The chain of lymph glands lying in the mesentery in close proximity to the caecum and ascending colon was found to have changed from the normal greyish colour and to have developed a dark red tint similar to the colour of the spleen. On section these glands were found to resemble haemolymph glands in structure and gave a marked iron reaction with  $K_4Fe(CN)_6$  and HCl. The lymphoid tissue had largely disappeared, leaving



open spaces crowded with phagocytic cells loaded with iron-reacting pigment and red blood corpuscles. In the more chronic cases there appeared to be proliferation of the reticulo-endothelial cells (figs. 5 and 6)

*Other lymph glands* Glands from the groin, axilla, neck and mediastinum showed similar changes. The lymphoid tissue had largely disappeared and the widely distended sinuses were full of red blood cells and macrophages loaded with hæmosiderin.

### *Control experiments*

Further experiments to test the effect of X-rays in producing these conditions were carried out in rats, mice and guinea-pigs without inoculation of any tumour.

1. *Rats* Twenty-four stock rats (nos 9-26 and 28-33 in table II) were irradiated in batches of 6 with 600, 500, 400 and 200 r respectively. Those receiving 200 r remained healthy and their red cell counts varied but slightly from the normal, only one showing a count diminished to 4,500,000 red cells, but an unaltered Hb content (colour index 1.6).

Two of the rats receiving 400 r were given 1000 r in addition and showed red cell counts of 5,400,000 and 5,250,000, but with Hb values of 100 per cent and 88 per cent respectively. None of these 6 rats died but 4 were subsequently killed—two after the initial dose of 400 r and two after 400 r + 1000 r—when their spleens were found to be atrophied with an average weight (in 3 animals) of 0.3 g.

Of the 6 rats receiving 500 r one died on the 20th day and was found too decomposed for section. The remaining five received a second dose of 1000 r, two dying with blood counts of 3,300,000 and 1,500,000 red cells and Hb of 23 and 10 per cent. Their spleens weighed 0.136 and 0.17 g and the mesenteric glands showed marked changes. The remaining three rats were killed and no considerable changes were found on examination.

Three of the six rats receiving 600 r had subsequently a second dose of 1000 r. Two of these showed a moderate fall in their red cell count, but when killed all six showed very slight changes in liver and glands.

No explanation can be given of the failure of X-radiation to cause death of most of the animals in this series, three only dying out of twenty-four.

General irradiation with 1200 r caused death in 4-5 days. Five Wistar rats receiving this dose were found dead before blood counts could be taken and were in too decomposed a state for section. Marked atrophy of lymphoid tissue was noted, however, only vestiges remaining in the mesentery.

## X RADIATION AND LYMPHOID TISSUE



FIG. 5—Lymphoid tissue in mesenteric gland of normal rat H and E  $\times 170$

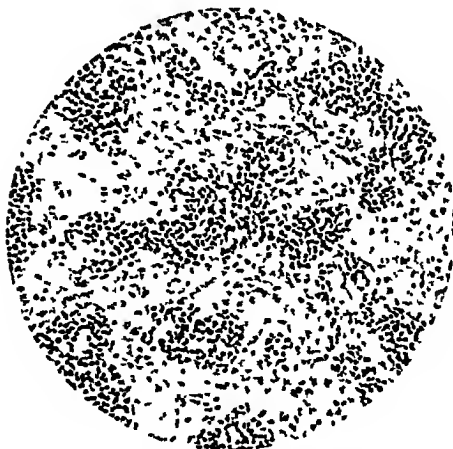


FIG. 6—Lymphoid tissue in mesenteric gland of irradiated rat showing diminution of lymphoid cells and sinuses loaded with macrophages H and E  $\times 170$



2 *Mice* These animals seemed more resistant to the effects of X-rays than rats and larger doses were used. Lethal doses were first employed, 1200 r causing the death of 20 mice in 3-5 days with symptoms of paralysis of the hind quarters. Blood counts showed in only one case a fall of the red cells to 3,000,000 (from the normal 8,000,000), six others giving only slightly diminished counts, but no fall in Hb was observed in any of the mice. The other mice showed increased red cell counts, and as they, like other generally irradiated animals, suffered from diarrhoea, the possibility of increased viscosity of the blood was entertained, but no estimations were made. Sections of glands, however, showed a change similar to that in irradiated rats, this being most marked in the glands of the groin and neck. No iron reaction was found in the thymus. The average weight of the spleen was 0.05 g (normal mouse spleen 0.16 g). Of 10 stock mice irradiated with 1000 r, 4 died in 7-11 days. In two of these, red cell counts were obtained of 1,000,000 and 5,000,000 before death, with a Hb content of 10 and 50 per cent respectively. The spleens weighed 0.05, 0.06, 0.06 and 0.07 g. Livers and kidneys showed marked fatty changes.

3 *Guinea-pigs* Six guinea-pigs irradiated with a lethal dose of 1500 r were found dying on the 5th day, and 4 irradiated with 1000 r also died within the same period. No blood counts were taken. The spleens averaged 0.165 g as compared with the normal weight of 0.8 g for a guinea-pig of 300 g. Fatty changes were advanced in the liver and kidneys, the lymphoid tissue was generally diminished and the prussian blue reaction was given by the mesenteric glands.

#### DISCUSSION

From the foregoing details it appears that death of the experimental animals was due to general irradiation and its far-reaching effects on the hæmopoietic tissues. In the case of the rats a *Bartonella* infection was suspected, but this was negatived by the following facts: (a) No *Bartonella* organisms were found in any of the experimental animals. (b) No leucocytosis was present, as is usual in this infection. The irradiation, however, may have prevented multiplication of the white cells. (c) The same condition of anaemia and the same pathological changes in the glands, liver, kidneys and spleen occurred also in mice and guinea-pigs when subjected to general irradiation. (d) With smaller doses of X-rays up to 300 r no mortality occurred amongst the rats. Had *Bartonella* been latent in these animals it seems probable that some at least would have succumbed to the infection. (e) Rats which remained healthy after general irradiation and were then subjected to a second lethal dose of 1000 r showed in

two cases sudden fatal anæmia consequent on the second dose. Had Bartonella been present it is probable that they would have succumbed to the initial irradiation of 500 r

A similar reaction with the appearance of profound anæmia and a fall to approximately the same level of red cell counts and Hb content as in general irradiation of rats has occurred in Amsterdam in patients with inoperable cancer subjected to general irradiation (personal communication by Dr Levine). Hæmorrhages from the mucous membranes were a feature of these cases, as in persons suffering from profound blood changes. Mallet (1936) reports, in general irradiation of cancer patients, that the effect on the red cells is to cause a drop in the total number, often to 2,600,000, at which point it is advisable to defer treatment, and states that 10 per cent of patients treated by this method showed symptoms of intolerance characterised by hæmorrhages and altered coagulation of the blood. In one case death supervened. It is also noteworthy that Gendreau and Pinsonneault (1937) describe the case of an X-ray worker suffering from a progressive anæmia in whom the red cell count fell before death to 1,632,000 per cmm with Hb content of 40 per cent. Histological examination showed fatty degeneration of the liver, diminution in the lymphoid tissue with numerous macrophages in the sinuses of the glands and spleen, and a marked iron reaction in the spleen with proliferation of the reticulo-endothelial cells.

Murphy and his collaborators (Murphy, 1926) have noted that irradiation causes a diminution in the number of lymphocytes in blood counts from experimental mice, and this has been interpreted as implying a break-down in the defensive mechanism of the lymphoid tissue, whereby tumour formation is favoured.

The erythrocytes of rats also appear to be greatly affected by lethal doses and no attempt at regeneration seems to take place except in animals which survive and in which normoblasts are to be found in the blood some 3-4 weeks after irradiation. In rats dying from the 5th to the 26th day the appearance of the anæmia is sudden, and the condition rapidly fatal. The animals die within a few hours though apparently well up to this point. Frequently rapid decomposition prevented examination of the organs and no terminal counts were taken. So rapid is the change that the red cell count, which falls but slowly in the first few days, falls in a few hours to one million or less, the terminal Hb estimations also declining with great rapidity and blood platelets disappearing from the blood. This condition is accompanied by equally rapid structural changes in the lymphoid tissue of the body, except that the thymus gland has not been noticed to show alteration beyond cloudiness of the cells and more faintly staining properties. The greatest change occurs in the mesenteric lymph nodes running up the ascending colon from the cæcum, and of these the gland at the junction of cæcum and colon appears most affected. Examination of the mesenteric glands from non-irradiated animals has in all cases shown normal lymphoid tissue and no excess of the reticulo-endothelial elements other than those usually seen in

the interstices of the glands, in no case was the prussian blue reaction given. But in animals dying from the effects of general irradiation, as in those surviving large doses and killed for examination, the reticulo-endothelial cells of the mesenteric nodes appear to have undergone proliferation at the expense of the lymphoid tissue, and a marked iron reaction is given. This disappearance of lymphoid tissue corresponds with the leucopenia apparent after irradiation, and the excess of reticulo-endothelial cells is probably the response to blood destruction taking place in the animal's body, the phagocytes being loaded with red cells and hæmosiderin and the general structure of the glands somewhat resembling that of the spleen. Both macroscopically and on section there seems to be a transformation of the normal lymph nodes into hæmolymph glands concerned with the disposal of broken down red cells. In the rat, hæmolymph glands occur along the renal vessels and along the splenic artery between spleen and stomach (Lewis, 1903). But examination of normal mesenteric glands in the caecal region reveals no hæmolymph structure, and it is only under the influence of X-rays (and, as will be seen later, under the action of a chemical compound) that this change becomes apparent. Not only the mesenteric lymph glands but also those of the groin, axilla and thorax show this transformation.

The fatty change in the liver and kidneys of generally irradiated animals would seem to be secondary to the profound anaemia and similar to conditions found in anaemias from other causes. The atrophy of the spleen which is known to occur in irradiated animals (Colwell and Russ, 1924) was a marked feature in the rats, mice and guinea-pigs subjected to general irradiation in these experiments.

The increased growth of the mouse sarcoma grafted into irradiated rats as compared with that grafted into non-irradiated rats suggests certain possibilities. It has usually been observed that animals in poor condition or suffering from intercurrent disease respond feebly to a grafted tumour, the growth of which is generally slow or the grafts may fail to take. But, as has been shown by Furth *et al* (1933) and by Clemmesen (1937), irradiated rats and mice are more susceptible to the induction of tumours than non-irradiated, and the tumours attain a larger size. The irradiated body appears to provide a more suitable environment for tumour growth. This applies equally to heterotransplanted transmissible tumours and to tumours produced experimentally by injection of a chemical compound. The obvious pathological change produced by X-rays is one affecting the hæmopoietic system by which the supply of lymphocytes is lessened, the red cells tend to be destroyed, unmasked iron is deposited in the tissues and the supply of oxygen is presumably defective. It is suggested that this deficiency in oxygen may be one of the factors necessary

to tumour growth Strong (1936) and Strong and Francis (1937) have also described a pre-malignant condition in dilute brown mice and in the Strong A strain, which develop carcinoma in old age, pointing out that prior to the appearance of the tumour a fall occurs in the Hb and blood protein levels. Non-susceptible mice such as the CBA do not show this fall. Similarly Orr (1937) is of opinion that ischaemia is conducive to the production of a malignant change in tissue.

The possibility that progressive blood destruction in the tissues is antecedent to tumour formation and a predisposing factor has been supported by experiments on an inbred strain of mice, the dilute brown. An attempt to obtain an experimental sarcoma in 10 of these mice by injection of the derivative of 1,2,5,6-dibenzanthracene named above proved unsuccessful, although doses of 0.25 c.c. of a 0.4 per cent solution were given thrice weekly for 218 days. The experiments were discontinued owing to infectious disease amongst the animals, the survivors being killed. On examination none gave evidence of tumour formation at the site of injection. In a second series, twenty mice of the same strain were employed, 15 of which had, previous to treatment with the compound, received general X-radiation in a single dose of 550 r. As in the first series, these and 5 non-irradiated controls were injected with similar amounts of the compound. The irradiated mice developed six tumours before the 225th day, the earliest obtained being on the 123rd day. Only one tumour has occurred amongst the 5 non-irradiated mice, which was first noticed on the 225th day. Fig. 7 shows the incidence of tumour formation in relation to time, the two series being combined for convenience.

The tumours obtained in the generally irradiated mice were all similar in type, being slow-growing sarcomas with a dense hard structure and scanty blood supply, which when grafted into other mice of the same strain gave 50 to 80 per cent. of takes. The tumour obtained from the non-irradiated mouse was encapsuled and contained soft haemorrhagic tissue, which on grafting into the dilute browns gave 100 per cent. of takes.

Examination of the lymph glands of the irradiated mice bearing these primary tumours showed iron-reacting deposits in the reticulo-endothelial cells. It was felt that possibly this change might be due to the irradiation received some months previously, but this was not supported by the fact that the lymphoid tissue of the non-irradiated mouse from which the haemorrhagic tumour was obtained also showed abundant iron-reacting deposits. Further, two non-irradiated mice of the CBA strain and one ordinary stock mouse which developed sarcomas after several months' treatment with the compound also showed considerable breaking down of blood in the lymph glands throughout the body. Mice and rats

treated with the chemical compound and killed at intervals during the latent period before tumour formation have also been examined. Iron-reacting deposits were found in the lymph glands of these

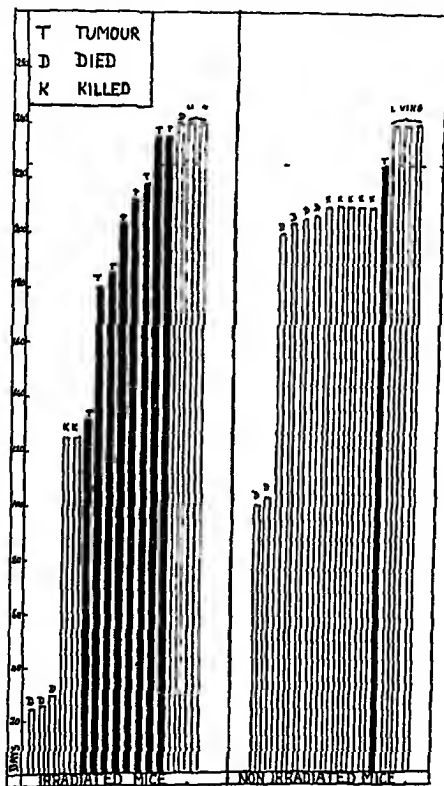


FIG 7—Chart showing incidence of tumours in a series of inbred mice undergoing treatment with a derivative of 1 2 5 6 dibenzanthracene. Each vertical line represents the duration of life of one mouse up to the date of detection of the tumour (block) or to the date when the mouse died or was killed. The dotted horizontal line at the 225th day serves to facilitate comparison.

animals. Normal controls are also being examined for similar changes, but so far, with the exception of one mouse which showed traces of iron in the groin glands, no evidence of breaking down of blood has been obtained.



Further investigations as to the presence of anæmia in animals suffering from spontaneous malignant growths and in those undergoing injections with the sarcoma-producing compound are being undertaken, with special reference to the abnormal presence of iron in the tissues prior to tumour formation and any evidence of a slow and hidden blood destruction

### SUMMARY.

1 The growth of a mouse sarcoma in generally irradiated rats was found to be more rapid and the tumour of larger size than in non-irradiated animals. Grafts into successive generations showed a higher percentage of takes in the irradiated rats than in the non-irradiated.

2 Investigation into the cause of the mortality of irradiated animals showed that general irradiation produces a diminution in the lymphoid tissue throughout the body, and in lethal or sub-lethal doses causes marked anæmia with structural changes in the lymph glands—more particularly the mesenteric, fatty changes in liver and kidneys and atrophy of the spleen.

3 The structural changes in the lymph nodes appear to convert these from normal lymph into hæmolymp glands concerned with phagocytosis of red cells.

4 The deposit of iron in the tissues of X-rayed animals and in those bearing primary tumours is discussed with special reference to the blood destruction found in the lymphoid tissue of irradiated and non-irradiated mice developing sarcomas after treatment with a chemical compound.

We are much indebted to the International Cancer Research Foundation for a personal grant to one of us (L. D. P.), and to the British Empire Cancer Campaign for grants which have supported this investigation.

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of a parenchymatous degeneration in which groups of tubules atrophy and disappear, leaving scar-like areas of fibrosis in their place. Other tubules become distended with albuminous coagulum so that the kidney as a whole is distorted with its surface pitted and granular (fig 1). In the more severe cases secondary changes such as hyaline degeneration of the glomeruli and arterioles may appear, and many glomeruli may be totally destroyed and obliterated. Even the arteries may appear thickened and tortuous, so that practically all the histological features of a human chronic nephritis may be reproduced. The condition offers an excellent opportunity for the study of fibrous tissue formation since it entails a replacement fibrosis, which can be followed through its different stages of development by killing off the animals at different periods.

The material upon which this study is based comprised more than 300 rats taken at various stages of the disease from the sixth to the six hundredth day, and showing various degrees of renal destruction.

### *Observations on the fibrotic process*

The development of the fibrosis is most easily followed in the renal cortex because there the interstitial tissue normally consists of little more than a delicate framework of reticulum, occupying a minimum space between the tubules. Collagenous material is seldom seen except in relation to the glomeruli and blood vessels, and when any appears amongst the tubules it may be regarded as pathological. In the medulla the interstitial tissue is normally more plentiful and mixed in character, consisting of both collagen and reticulum, so that to distinguish new-formed collagen from the original interstitial tissue in this region is less easy. For the study of interstitial tissue in the kidney I have found the Bielschowsky-Foot technique, using van Gieson as a counterstain (Foot and Ménard, 1927), the most valuable.

In the rat nephritis interstitial fibrosis arises by two different processes, one consisting of a condensation and transformation of the original reticular framework of the kidney, the other of an actual new formation of tissue. The first process may be described in a few words. When tubules atrophy and disappear the network of reticulum which encloses them collapses so as to form dense masses of fibrils in which endothelial and other cells, including degenerate remnants of epithelium, may be crowded together (figs 2 and 3). In sections stained with haemalum and eosin, such masses have the appearance of fibrous scars, but with special connective tissue stains they are usually shown to contain no actual new formation of tissue, merely a change in the arrangement and distribution of the old. It is only in the later stages of this process that collagen appears, and then it seems to arise simply by a transformation of the reticulum fibres. In the early stages these fibres remain distinct and discrete, but later they lose their



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## GENESIS OF RENAL FIBROSIS



FIG. 5.—Kidney of rat which received  $\text{NaH}_2\text{PO}_4$  and 40 000 units of calciferol for 25 days and subsequently lived 101 days. The interstitial spaces are swollen with hyaline material. Haemalum and eosin  $\times 200$ .



FIG. 6.—Same case as in fig. 5 showing an area of dense fibrosis probably representing a destroyed glomerulus. The reticular fibres at the margins of the area are stained black with silver but in the area itself they are no longer differentiated.  $\times 200$ .



FIG. 7.—Same case as in fig. 4 showing hyaline degeneration of a glomerulus.  $\times 200$ .



FIG. 8.—Kidney of rat which received  $\text{NaH}_2\text{PO}_4$  and 40 000 units of calciferol for 25 days and subsequently lived 50 days showing a glomerulus and its arteries. Haemalum and eosin  $\times 200$ .

substance is itself changed into collagen and actually forms the new connective tissue

In the presence of this change, the reticulum fibres may at first remain distinct, although they are usually separated into finer strands by infiltration of the hyaline ground substance into their interstices. Later, when the ground substance has become condensed and pink-staining, they tend to lose their argyrophil character so that they also take the pink of the van Gieson and are no longer clearly distinguishable from the rest of the interstitial substance (fig. 6), except for the somewhat fibrillated appearance which they sometimes impart to it. Thus in the end both the original reticulum and the new-formed ground substance combine to form pink-staining masses of collagen.

The relationship of this process to the interstitial cells is the point on which my attention has been especially focussed. In the early stages of the nephritis there is sometimes, along with the oedema, an infiltration of round cells into the tissue spaces as well as an increase of endothelial and other cells, and the question whether any of these are responsible for the new formation of connective tissue is the main issue. The answer can as yet only be offered as a matter of opinion, but there would seem to be little reason to regard the two as essentially related. Although the cellular increase and the new formation of tissue may often coincide, their relative proportions are by no means constant and in fact the interstitial substance is often found in greatest quantities when there are few cells (fig. 5). It is true that the cells and fibrils are often arranged in a rough alignment such as might suggest that they are growing in conjunction, and Wolbach (1933) has cited this common feature as evidence in favour of the cellular hypothesis, but the arrangement is in my opinion of little significance. The fibril formation is due in the first place to the original reticulum, which, although it becomes dedifferentiated so far as staining is concerned, retains its fibrillary form, and the cells which lie amongst it will naturally adjust themselves to this by arranging themselves in alignment with the fibrils. The hyaline ground substance which seems to form the basis of collagen is a homogeneous material apparently diffused through the tissues and more like a fluid infiltration than a formed product of any particular cell or group of cells. Its nature is unknown, but everything in its appearance seems to suggest an inflammatory or degenerate product of an exudative character.

Further light on fibrous tissue formation is sometimes afforded by the glomerular degeneration which appears in the more severe cases. Many of the glomeruli are totally destroyed and their ultimate state is fibrosis. They first undergo a kind of hyaline degeneration, their capillary walls becoming swollen and fused

## GENESIS OF RENAL FIBROSIS



FIG 5—Kidney of rat which received  $\text{NaH}_2\text{PO}_4$  and 40 000 units of calciferol for 25 days and subsequently lived 161 days. The interstitial spaces are swollen with hyaline material. Hemalum and eosin  $\times 200$



FIG 6—Same case as in fig 3 showing an area of dense fibrosis probably representing a destroyed glomerulus. The reticular fibres at the margins of the area are stained black with silver but in the area itself they are no longer differentiated  $\times 200$



FIG 7—Same case as in fig 4 showing hyaline degeneration of a glomerulus  $\times 200$



FIG 8—Kidney of rat which received  $\text{NaH}_2\text{PO}_4$  and 40 000 units of calciferol for 25 days and subsequently lived 50 days showing a glomerulus and its arterioles completely fibrosed. Hemalum and eosin  $\times 200$



and their capsular spaces obliterated. Later, their cells fade and disappear until nothing is left but masses of hyaline material (fig 7). At first these stain yellow with van Gieson but later they take a pink colour, so that they have the appearance of collagen. For a time these remnants of glomeruli remain clearly distinguishable as dense fibrous bodies (fig 8) having a somewhat convoluted internal structure, but later they shrink and become more compact while their outlines become less distinct, until finally they merge with the surrounding connective tissue and are lost. In this process there is no sign of any removal of tissue or substitution, simply a dedifferentiation and transformation into collagen.

A similar process may sometimes be seen in degenerate arterioles and even in tubules. When the tubules atrophy they collapse and form solid cords of cells which gradually disappear. There is nothing to suggest that their disappearance involves phagocytosis or absorption. The cells merely lose their nuclear staining and, becoming homogeneous, take first a yellow and later a pink colour with van Gieson. Ultimately they become uniformly pink so that they are no longer distinguishable from the surrounding interstitial tissue, and thus it would seem as if the cytoplasm were itself actually changed into collagen. Apparently therefore degenerate tissue of almost any kind, including destroyed glomeruli and atrophic epithelium, may provide the material from which fibrous tissue is formed.

#### *Discussion*

Since the middle of last century the current views on connective tissue formation have been strongly influenced by the cellular hypothesis. Some authorities have held that the fibres are formed in the cell protoplasm, others that they are formed from the fluid intercellular substance which is itself a secretion of the cell, but nearly all have agreed in regarding the cell as the essential tissue builder. It is only recently that the idea of connective tissue arising from a substance of non cellular origin has been to any extent entertained.

In 1910 Hertzler concluded from a study of organisation in the peritoneum that fibrous tissue formation was comparable with fibrin formation in blood coagulation, the cell taking no active part in the process. Later Bartsell (1916) described the transformation of fibrin into connective tissue in wound organisation at regions where no cells were present, and he also (1915) described fibril formation in the plasma medium in tissue cultures. Alfjow (1925, 26) and Harrison (1924, 25) considered that fibres, in embryonic development, arose from an amorphous ground substance without any direct connection with cells, and Foot and Day (1925) suggested that fibre formation in tumours was something in the nature of a precipitation or accretion in the intercellular ground substance. This has been largely corroborated by Maximow (1928) from his observations on tissue culture. In a study of nerve regeneration Nageotte (1922) concluded that various materials including dead and degenerate tissues and cells may go to the formation of

collagenous connective tissue, and recently Day (1936) has ventured the suggestion that the oedema fluid may provide the natural substrate for the fibrous tissue formation which occurs in chronic lymphatic obstruction. Further support has been lent to the non-cellular theory by the work on tissue culture of Doljanski and Roulet (1933) and von Jeney and Törö (1936-37). These writers have described fibres developing in the culture medium at parts distant from the living cells and have concluded that their formation is a colloidal chemical reaction rather than a cytological one. The latter authors believe that a specific substance, probably identical with ascorbic acid, is the essential agent in this reaction.

There is thus a considerable body of opinion favouring the non-cellular theory of connective tissue formation, nevertheless, in so far as the pathology of renal disease is concerned, the cellular hypothesis still prevails, and most authorities regard interstitial fibrosis as a proliferative phenomenon.

While it is admitted that histology may mislead there are some points of evidence in the rat nephritis which it would seem fairly safe to accept. There is evidence for example that fibrous tissue is formed by a transformation of the original reticulum. It is generally acknowledged even by observers who adhere to the cellular hypothesis (*e.g.* Mallory and Parker, 1927) that such a transformation may occur, and there are in fact many authorities who regard reticulum as the natural precursor of collagen. This alone is strong evidence against the cellular hypothesis because, as has been pointed out, the fibrosis in milder cases of the rat nephritis may be nothing more than a condensation and differentiation of the original reticulum. In the more severe cases the obliteration of glomeruli is a simple fibrous transformation in which the cells seem to play an entirely passive role. Thus, leaving all other points out of consideration, there is evidence enough that renal fibrosis may occur independently of cells. There is no doubt that other processes may contribute, and I have described above a hyaline ground substance which I regard as the basis of much of the new formation of collagen, but as yet little is known of the nature of this ground substance and the question of its importance in tissue formation is left for further investigation. The important point in the rat nephritis is the total absence of evidence that connective tissue arises from any particular cell, or that it depends on the proliferative activity of any cells. All the evidence on the contrary seems to point to the fibrosis as a degenerative or retrogressive phenomenon rather than a proliferative one.

In describing this experimental lesion in rats I have used the term "nephritis" because I regard the changes as differing in no important essential from those seen in certain types of the human disease bearing that name. The two lesions correspond histologically there seems no reason to suppose that they differ in their mode of origin. In the rat nephritis fibrosis appears to be the

natural process whereby the kidney disposes of tissues which are no longer serving their natural purpose, and in view of this it would seem that the prevailing conception of interstitial nephritis as a reactive cellular change requires to be revised

### Conclusions

The development of pathological fibrosis has been studied in an experimental nephritis in rats produced by the oral administration of acid sodium phosphate and calciferol

No evidence has been found to support the cellular theory of connective tissue formation or to suggest that interstitial cell proliferation is an essential factor in renal fibrosis

Renal fibrosis seems to be produced mainly by the collapse and condensation of the original interstitial framework of the kidney around atrophic tubules with subsequent transformation of the reticulum and other tissues into collagen

In some cases an infiltration of a hyaline ground substance into the tissue spaces is seen and this also appears to form the basis of new connective tissue formation

All the evidence seems to point to renal fibrosis as a degenerative or retrogressive rather than a proliferative phenomenon

Part of this work was assisted by a grant from the Medical Research Council

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collagenous connective tissue, and recently Day (1936) has ventured the suggestion that the oedema fluid may provide the natural substrate for the fibrous tissue formation which occurs in chronic lymphatic obstruction. Further support has been lent to the non-cellular theory by the work on tissue culture of Doljanski and Roulet (1933) and von Jeney and Törő (1936-37). These writers have described fibres developing in the culture medium at parts distant from the living cells and have concluded that their formation is a colloidal chemical reaction rather than a cytological one. The latter authors believe that a specific substance, probably identical with ascorbic acid, is the essential agent in this reaction.

There is thus a considerable body of opinion favouring the non-cellular theory of connective tissue formation, nevertheless, in so far as the pathology of renal disease is concerned, the cellular hypothesis still prevails, and most authorities regard interstitial fibrosis as a proliferative phenomenon.

While it is admitted that histology may mislead there are some points of evidence in the rat nephritis which it would seem fairly safe to accept. There is evidence for example that fibrous tissue is formed by a transformation of the original reticulum. It is generally acknowledged even by observers who adhere to the cellular hypothesis (*e.g.* Mallory and Parker, 1927) that such a transformation may occur, and there are in fact many authorities who regard reticulum as the natural precursor of collagen. This alone is strong evidence against the cellular hypothesis because, as has been pointed out, the fibrosis in milder cases of the rat nephritis may be nothing more than a condensation and de-differentiation of the original reticulum. In the more severe cases the obliteration of glomeruli is a simple fibrous transformation in which the cells seem to play an entirely passive role. Thus, leaving all other points out of consideration, there is evidence enough that renal fibrosis may occur independently of cells. There is no doubt that other processes may contribute, and I have described above a hyaline ground substance which I regard as the basis of much of the new formation of collagen, but as yet little is known of the nature of this ground substance and the question of its importance in tissue formation is left for further investigation. The important point in the rat nephritis is the total absence of evidence that connective tissue arises from any particular cell, or that it depends on the proliferative activity of any cells. All the evidence on the contrary seems to point to the fibrosis as a degenerative or retrogressive phenomenon rather than a proliferative one.

In describing this experimental lesion in rats I have used the term "nephritis" because I regard the changes as differing in no important essential from those seen in certain types of the human disease bearing that name. The two lesions correspond histologically and there seems no reason to suppose that they differ in their mode of development. In the rat nephritis fibrosis appears to be the

# THE ACTION OF SODIUM POLYANETHIOL SULPHONATE ("LIQUOID") ON BLOOD CULTURES

T. VON HALDLER and A. A. MILES

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The anticoagulant "Liquoid,"\* a synthetic polymer of sodium anethiol sulphionate, has been used on the continent as an adjunct to blood culture for the past five years.

It is reported to be a stable compound, unchanged by heating or by the action of dilute acids and alkalis, at least as effective as hirudin as an anticoagulant, acting in this capacity as other aromatic compounds of high molecular weight, anticomplementary and destructive of the bactericidal power of human, rabbit and guinea pig blood, the latter effect being due in part to the inhibition of the  $\beta$  lysins of the serum (Battistini, 1932, De Antoni and Cartalam, 1933, both quoted by Grulichova, 1935, van der Hoeven, 1937). By its action, fresh blood is converted into a favourable culture medium for a large number of micro organisms, including *B anthracis*, *Br melitensis* and *abortus*, *H influenza*, *L icterohaemorrhagiae*, *N gonorrhoeae* and *meningitidis*, *Staph aureus* and *albus*, *Str haemolyticus*, *pneumoniae* and *viridans*. The coli typhoid organisms are also favoured, but not so consistently (Karlsb, 1936, Massa and Battistini, 1934, Niesner and Valencova, 1934, Friedmann, 1936, Sgalitzer, 1936, Grulichova, 1935, Gara and Sogliani, 1936, quoted by van der Hoeven, 1937). Liquoid may be added directly to withdrawn blood or incorporated into liquid culture media in concentrations of 0.1-0.2 per cent.

This paper reports an investigation of the action of liquoid in modifying the capacity of defibrinated normal human blood to support the growth of pathogenic bacteria, with a view to assessing its value as an anticoagulant for use in routine blood culture.

## Materials and methods

The strains of bacteria used in the tests were, where possible, freshly isolated. The recently isolated and stock strains were all S forms.

The following cultures were used: *B anthracis*, NCTC 109, recent guinea pig passage; *Bact typhosum* rejuvanted Rawlings strain *Bact friedländeri*, NCTC 204; *Bacteroides* strain, Gram negative non pathogenic obligate anaerobe from normal human faeces; *Br melitensis* Palestine, Yonkovitch 6; *Cl welchii*, NCTC 114; *H influenza*, Straker L85, equivalent to Pittman's smooth form; *H para influenza*, 7994, from fatal case of endocarditis; *N meningitidis*, strain 1, NCTC 3712, strain 2, C150 freshly isolated from CSF; *N gonorrhoeae*, strain 1, SG213.

\* Liquoid is the proprietary name given to this compound by its manufacturers Messrs Hoffmann La Roche.



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strain 2, SG294; both recently isolated from vaginal discharge *N. pharyngis*, freshly isolated from naso-pharynx *Proteus vulgaris*, U756, from pure urinary infection *Ps. pyocyanea*, from pus. *Staph aureus*, P728, from pus *Staph albus*, freshly isolated from skin *Str. haemolyticus*; Lancefield's group A *Str pneumoniae*, Type I, recent mouse passage *Str. viridans*, recently isolated from throat Anaerobic streptococcus; P542, recently isolated from pleural empyema Aerobic organisms were used as 24-hour cultures, anaerobes after 48 hours' incubation

The blood was obtained from, in all, 12 healthy human adults, male and female, but for the most part the tests were made on blood from one individual This was defibrinated, filtered through one layer of sterile gauze, distributed into  $3 \times \frac{1}{2}$  inch tubes in 1 c c quantities and used immediately. To the blood samples were added broth, anticoagulants and dilutions of culture as required. Liqueid, 5 per cent. in normal saline, was autoclaved for 10 minutes at  $115^{\circ}\text{C}$ , sodium citrate, 50 per cent in distilled water, for 20 minutes at  $115^{\circ}\text{C}$  Liquor trypsin (Allen and Hanbury's) was sterilised by filtration through Seitz E.K. filters.

Three 10-fold dilutions of culture, selected as likely to contain about 5, 50 and 500 viable organisms, were inoculated into three blood tubes for each test The actual number of viable organisms inoculated was estimated from the mean of a six-plate "surface viable" count (Aitken, Barling and Miles, 1936) made upon each culture Organisms which emulsified with difficulty, e.g. the gonococcus, *H. para-influenzae*, and *N. pharyngis*, were dispersed in broth as much as possible, and the bacterial aggregates removed by light centrifugation Blood mixtures containing aerobes were tested for growth after 24 hours, anaerobes after 48 and 96 hours The degree of growth was estimated at first by surface viable counts and later by direct plating of one loopful of culture on to an appropriate medium and by inoculating 0.25-0.5 c c into 5 c c of heart broth Broth subcultures showing no obvious growth after 48 hours were tested by further plating. The medium used for the counts and tests with the typhoid and anthrax bacilli, the staphylococci and *Ps. pyocyanea* was nutrient agar. Fildes' agar was used for the *Haemophilus* group, haemoglobin agar for the meningococci and gonococci (in 5 per cent  $\text{CO}_2$ ), MacConkey's agar for *Proteus* and 5 per cent horse-blood agar for the rest

The tables record, in organisms per c c, either the smallest inoculum which multiplied, or, if none of the inocula survived, the largest inoculum which failed to do so The degree of growth after 24 hours (48 hours for the anaerobes) is indicated in the tables either by the result of a viable count (organisms per c c) or by the following signs. + + +, free growth on the test plate; + +, semiconfluent growth on the test plate, +, discrete colonies on the test plate;  $\pm$ , no growth on plate, growth in the broth subculture, —, no growth in the broth subculture after 2 days for aerobes and 4 days for anaerobes

## RESULTS.

A preliminary test was made, incorporating three anticoagulants, sodium citrate, liquor trypsin and liqueid, in solid media, which were tested with 10-fold dilutions of cultures of *Str pneumoniae* and *Br melitensis* (upon blood agar) and of *H. influenzae* and *Staph aureus* (upon Fildes' agar). Compared with the growth on untreated media, 0.75 per cent sodium citrate partly inhibited *Br melitensis* and completely inhibited an inoculum of  $15 \times 10^4$  *H. influenzae*,

4 per cent liquor trypsin partly inhibited *H influenza* and completely inhibited an inoculum of 1900 *Str pneumonia*. The remaining strains were unaffected. The growth of *Staph aureus*, *Str pneumonia*, and *Br melitensis* was, if anything, improved by 0.1 and 0.5 per cent liquor, but *H influenza* was markedly inhibited by these amounts.

### *Tests on defibrinated blood*

For the first tests on 1 c.c. quantities of fluid blood, 0.2 per cent liquor was used. The test on each organism was made twice, in some cases three times, with substantially the same results. Table I (columns 2-5) exemplifies these results. The first four organisms tested grow well in blood and liquor-blood. The next group comprises those that failed to grow or established themselves only from relatively large inocula in blood, but grew freely in liquor-blood. Of the third group, the two anaerobes, *Cl welchii* and the *Bacteroides* strain, failed to grow in either blood or liquor-blood (in view of the later tests, this was probably due to a technical error), *N gonorrhoeae* grew better in liquor-blood but was partly inhibited by it, while the *N meningitidis* strains were markedly inhibited by liquor.

This susceptibility of the meningococcus to liquor appears in Grubb's table IV, which records an inoculum of 680 meningococci in 0.17 per cent liquor-blood decreasing to 280 in 24 hours. Our meningococcal strains were retested in 0.1, 0.05 and 0.025 per cent liquor-blood. In the 0.1 per cent both strains were inhibited to a certain extent, but in the 0.05 and 0.025 per cent, 10 organisms increased to  $1.25 \times 10^6$  and  $9.0 \times 10^6$  respectively in 24 hours. To determine the minimal effective anticoagulant concentration, three specimens of normal human blood were tested with 0.05, 0.025 and 0.0125 per cent liquor, the two higher concentrations inhibited clotting for at least 7 days, while in blood containing 0.0125 per cent a loose clot formed in four hours, which was easily and permanently dispersed by gentle shaking.

A concentration of 0.05 per cent liquor was selected as being actively anticoagulant and non-inhibitory of *N meningitidis*. Table I (columns 6-9) shows the result of a retest with this concentration. *Br melitensis*, *Str haemolyticus A* and *pneumonia*, and the *Bacteroides* grew as well in blood as in liquor-blood. The remaining strains were all favoured by 0.05 per cent liquor.

### *Comparison of broths containing liquor and liquor trypsin*

Trypsin broth, made by adding 5 per cent sterile Seitz-filtered liquor trypsin to heart extract broth, is an excellent blood culture medium. Its manufacture demands fresh trypsin solution, laborious

TABLE I.—Effect of liquid on growth of bacteria in 1 c.c. defibrinated blood.

Organisms	Without liquid		With liquid 0.2 per cent		Without liquid		With liquid 0.05 per cent	
	Approximate number inoculated	Result	Approximate number inoculated	Result	Approximate number inoculated	Result	Approximate number inoculated	Result
<i>B. anthracis</i> .	14	±	14	200	10	±	10	±
<i>Staph aureus</i> .	3	++	3	++	200	++	200	++
<i>Streptococcus A</i> .	20	0 × 10 <sup>5</sup>	20	11 × 10 <sup>5</sup>	2	++	2	++
<i>Streptococcus B</i> .	2	—	2	—	10	++	10	++
<i>Streptococcus C</i> .	3	++	3	++	10	++	10	++
<i>B. anthracis</i> .	2100	—	14	++	160	—	16	++
<i>Bact. fecalibacter</i> .	200	—	3	++	200	—	20	++
<i>Bact. typhosum</i> .	600	—	6	++	150	—	15	++
<i>B. influenzae</i> .	1600	—	16	++	1600	—	160	++
<i>B. para-influenzae</i> .	2200	—	2	30 × 10 <sup>5</sup>	—	—	—	—
<i>N. meningitidis</i> .	700	—	2	142 × 10 <sup>5</sup>	—	—	—	—
<i>N. meningitidis</i> .	70	±	7	±	550	—	55	++
<i>N. meningitidis</i> .	2500	—	25	++	2000	—	200	++
<i>Proctus vulgaris</i> .	100	—	9	++	100	—	10	++
<i>P. pyocyanea</i> .	2000	±	200	++	500	—	50	++
<i>Staph. albus</i> .	200	—	10	++	100	—	100	++
<i>Streptococcus (anaerobius) *</i> .	910	—	11	++	1700	—	20	++
<i>Streptococcus</i> .	110	—	20	++	3000	—	300	++
<i>Streptococcus</i> .	2000	—	—	—	—	—	—	—
<i>N. meningitidis</i> .	3600	—	360	+	150	—	15	++
<i>Bacteroides *</i> .	3600	—	36	—	25	++	25	++
<i>C. welchii *</i> .	90	—	90	—	140	++	14	++
<i>N. meningitidis</i> .	53	++	1800	—	14	—	20	++
<i>N. meningitidis</i> .	4	—	—	—	200	++	10	++
<i>N. meningitidis</i> .	20	++	—	—	100	++	—	—
<i>N. meningitidis</i> .	2	—	35	—	10	++	—	—

\* 48 hours' incubation

± to ++ = varying amounts of growth (text, p. 246)

— = no growth

sterile manipulations and subsequent sterility tests, and it must be used freshly made. It was one of the objects of this investigation to determine whether liquoid-broth might be an efficient substitute. Parallel tests were made with 100 quantities of 0.05 per cent liquoid heart broth, and 8 per cent liquor trypsin broth, to each of which was added 0.5 c.c. of defibrinated blood, giving a final concentration of approximately 0.03 per cent liquoid and 5 per cent liquor trypsin respectively. Table II summarises the results of

TABLE II

Effect of liquoid and trypsin on growth in blood broth

Organisms	Defibrinated blood 0.5 c.c. + heart broth 1 c.c.					
	Without liquoid or trypsin		With liquoid (0.03 per cent.)		With liquor trypsin (5 per cent.)	
	Approximate number inoculated	Result.	Approximate number inoculated	Result	Approximate number inoculated	Result
<i>B anthracis</i>	15	+++	15	+++	15	+++
<i>Bact friedländeri</i>	100	—	10	+++	10	+++
<i>Bact typhosum</i>	15	+++	15	+++	15	+++
<i>Bacteroides</i> *	3500	++	3500	+++	3500	+++
<i>Br melitensis</i>	10	++	10	+++	10	+++
<i>Cl welchii</i> *	2	+++	2	+++	2	+++
<i>H influenza</i>	60	—	6	+++	6	+++
<i>N gonorrhoeae</i> 1	50	++	50	+++	50	+++
<i>N gonorrhoeae</i> 2	15	+	15	+++	15	+++
<i>N meningitidis</i> 1	30	+++	30	+++	30	+++
<i>N meningitidis</i> 2	5	+++	5	+++	5	+++
<i>N pharyngis</i>	70	+++	70	++	70	++
	7	—	7	++	7	—
<i>Proteus vulgaris</i>	50	+++	5	+++	5	+++
	5	—	5	+++	5	+++
<i>Ps pyocyanea</i>	60	+++	6	+++	0	+++
	6	—	6	+++	0	+++
<i>Staph albus</i>	200	++	20	+++	20	+++
	20	—	20	+++	20	+++
<i>Staph aureus</i>	20	+++	20	+++	20	+++
<i>Streptococcus</i> (anaerobic)*	70	+	7	+++	7	+++
	7	—	7	+++	7	+++
<i>Str hemolyticus</i> A	3	+++	3	+++	3	+++
<i>Str pneumoniae</i> I	0	+++	0	+++	0	+++
<i>Str viridans</i>	50	+++	5	+++	5	+++
	5	—	5	+++	5	+++

\* 48 hours incubation.

this test. Both media were equally good in inhibiting the bactericidal power of the bloods tested, except in their behaviour towards *N pharyngis*, which was partly inhibited by the trypsin. The improvement with either anticoagulant is not as marked in this as in previous experiments, the bactericidal power of the bloods used in this experiment was small.



TABLE I.—Effect of liquid on growth of bacteria in 1 c.c. defibrinated blood.

Organism	Without liquid		With liquid 0.2 per cent		Without liquid		With liquid 0.05 per cent	
	Approximate number inoculated	Result	Approximate number inoculated	Result	Approximate number inoculated	Result	Approximate number inoculated	Result
<i>Br. melitensis</i> .	14	±	14	200	10	±	10	±
<i>Staph. aureus</i> .	3	+	3	+	200	+	200	+
<i>Str. hemolyticus</i> A	20	9 × 10 <sup>5</sup>	20	11 × 10 <sup>5</sup>	2	+	2	+
	2	—	2	—	10	+	10	+
<i>Str. pneumoniae</i> 1	3	+	3	+				
<i>H. influenzae</i> .	2100	—	13	+	160	—	16	+
<i>Bact. fragilis</i> .	200	—	2	+	200	—	20	+
<i>Bact. typhosum</i> .	660	—	6	+	150	—	15	+
<i>H. influenzae</i> .	1600	—	16	+	1600	—	160	+
<i>H. parva influenzae</i>	2200	—	2	30 × 10 <sup>5</sup>				
<i>N. gonorrhoeae</i> 2	780	—	2	142 × 10 <sup>5</sup>				
	70	±	7	±	550	—	55	+
<i>N. pharyngis</i> .	2500	—	25	+	2000	—	200	+
<i>Proteus vulgaris</i> .	900	—	9	+	100	—	10	+
<i>Pr. pyocyanea</i> .	2000	±	200	+	500	—	50	+
	200	—	10	+	100	—	100	+
<i>Staph. albus</i> .	910	—					10	—
			14	+	1700	—	20	+
<i>Streptococcus (aerobius)</i> *	140	—	20	+	3000	—	300	+
<i>Str. viridans</i> .	2000	—						
<i>N. gonorrhoeae</i> 1	3600	—	360	+	150	—	15	+
<i>Bacteroides</i> *	3600	—	36	—	25	+	25	+
<i>C. ueleni</i> *	90	—	90	—	140	+	14	+
<i>N. meningitidis</i> 1 .	56	+	1800	—				
	4	+			200	+	20	+
<i>N. meningitidis</i> 2	20	+			100	+		
	2	—	35	—	10	—	10	+

\* 48 hours' incubation

± to + + + = varying amounts of growth (text, p. 216)

— — — no growth

(a) The strain of *B anthracis* was killed with equal rapidity in whole blood, fresh serum and serum heated to 56° C for 20 minutes, this bactericidal action was inhibited by 0.074 per cent. liquoid.

(b) The strain of *Proteus vulgaris* was killed with equal rapidity by whole blood and fresh serum, but heated serum was bacteriostatic only both bactericidal and bacteriostatic powers were inhibited by 0.074 per cent liquoid.

These experiments were twice repeated and, in all, blood from three persons was tested, with the same results.

The superiority of liquoid over citrate in allowing the growth of organisms is indicated in our preliminary experiments incorporating the anticoagulants in solid media, and has been demonstrated by Gara and Soghian (quoted by van der Hoeden), Grilichess, van der Hoeden, and Sgalitzer. The last named, working mainly with blood from patients with a *Str viridans* bacteraemia, found in addition that oxalic acid was as ineffective as citrate, and that 1 per cent saponin-broth was slightly better than 0.17 per cent liquoid-blood or 0.09 per cent liquoid-broth. The action of saponin in this respect needs confirmation, it is possible that the comparative inferiority of liquoid in Sgalitzer's experiments was due to its use in a concentration of 0.17 per cent, which we have demonstrated to be inhibitory to the growth of certain bacteria under our experimental conditions. The comparatively unfavourable effect of liquoid in blood cultures of the coli-typhoid group noted by various authors may also have been due to the use of inhibitory concentrations. So far as our test organisms are concerned, a concentration in the blood of 0.05 per cent is safe, and is about four times the minimal effective anticoagulant concentration. *N meningitidis* appears to be the most suitable organism for determining the growth-inhibitory concentration of liquoid.

The growth of bacteria in normal defibrinated human blood under our test conditions is not strictly comparable with their growth in media containing the blood from which it is hoped to isolate them, but the results obtained suggest that the use of liquoid media in blood culture deserves consideration. Usually one strain of a given species has been tested and at most two. There are no extensive data upon the variability of different strains of the same species of bacteria in their susceptibility to liquoid, nor is it known whether there are antibacterial substances in the blood which liquoid may be unable to counteract, there is need for tests upon a large number of freshly isolated strains of the different pathogenic bacteria.

With regard to the substitution of liquoid-broth for trypsin-broth, though there is little demonstrable difference in the growth-promoting properties of the two, the less elaborate technique of preparation of the former renders it the more convenient routine medium.

*The effect of liquoid on whole blood*

The bloods used in the above test were fibrin-free. It is possible that, when liquoid is added to whole blood, some is used in maintaining the uncoagulated state, with a consequent lessening of the antibactericidal powers of the given concentration. A comparison was made of a whole blood, kept fluid with 0.2 per cent liquoid, and the same blood defibrinated, to which 0.2 per cent liquoid was added after defibrination. The test organisms were *H. influenzae*, *Br. melitensis* and *Proteus vulgaris*. Table III shows

TABLE III.

*Effect of liquoid on growth of bacteria in whole and defibrinated blood*

Organisms	Approximate number inoculated	0.2 per cent liquoid-blood	
		Defibrinated	Whole
<i>Br. melitensis</i>	30	+	+
	3	+	+
<i>H. influenzae</i>	15	+++	+++
	1-2	++	+
<i>Proteus vulgaris</i>	12	++	+++
	1-2	++-	+

the results. As far as the ultimate growth is concerned, there is no difference between the two bloods, but very small inocula of *H. influenzae* and *Proteus vulgaris* grow less vigorously in the whole blood.

*The stability and keeping power of liquoid-broth*

The efficacy of 0.2 per cent liquoid broth, made by autoclaving the mixture at 115° C. for 30 minutes, was compared with that of broth autoclaved separately, to which sterile liquoid, autoclaved at 115° C. for 10 minutes, was subsequently added. Broth and defibrinated blood were mixed in 0.5 cc quantities and tested with *Br. melitensis*, *H. influenzae* and *Proteus vulgaris*. The media were stored at 2° C. and the test was repeated after 3 weeks and again after 2 months. The results were identical in all three tests, indicating that neither autoclaving in broth nor storing for two months alters the antibactericidal or anticoagulant properties of liquoid.

## DISCUSSION

The experiments recorded above demonstrate the capacity of liquoid to inhibit the bactericidal action of normal human blood. We have not investigated its mode of action, but the results of the two following experiments are in conformity with the hypothesis that it neutralizes the effects both of complement and of  $\beta$ -lysin.

(a) The strain of *B anthracis* was killed with equal rapidity in whole blood, fresh serum and serum heated to 56° C for 20 minutes, this bactericidal action was inhibited by 0.074 per cent liquorid

(b) The strain of *Proteus vulgaris* was killed with equal rapidity by whole blood and fresh serum, but heated serum was bacteriostatic only; both bactericidal and bacteriostatic powers were inhibited by 0.074 per cent liquorid

These experiments were twice repeated and, in all, blood from three persons was tested, with the same results

The superiority of liquorid over citrate in allowing the growth of organisms is indicated in our preliminary experiments incorporating the anticoagulants in solid media, and has been demonstrated by Gara and Sogliani (quoted by van der Hoeden), Grihchess, van der Hoeden, and Sgalitzer. The last named, working mainly with blood from patients with a *Str viridans* bacteraemia, found in addition that oxalic acid was as ineffective as citrate, and that 1 per cent saponin-broth was slightly better than 0.17 per cent liquorid-blood or 0.09 per cent liquorid-broth. The action of saponin in this respect needs confirmation, it is possible that the comparative inferiority of liquorid in Sgalitzer's experiments was due to its use in a concentration of 0.17 per cent, which we have demonstrated to be inhibitory to the growth of certain bacteria under our experimental conditions. The comparatively unfavourable effect of liquorid in blood cultures of the coli-typhoid group noted by various authors may also have been due to the use of inhibitory concentrations. So far as our test organisms are concerned, a concentration in the blood of 0.05 per cent is safe, and is about four times the minimal effective anticoagulant concentration. *N meningitidis* appears to be the most suitable organism for determining the growth-inhibitory concentration of liquorid.

The growth of bacteria in normal defibrinated human blood under our test conditions is not strictly comparable with their growth in media containing the blood from which it is hoped to isolate them, but the results obtained suggest that the use of liquorid media in blood culture deserves consideration. Usually one strain of a given species has been tested and at most two. There are no extensive data upon the variability of different strains of the same species of bacteria in their susceptibility to liquorid, nor is it known whether there are antibacterial substances in the blood which liquorid may be unable to counteract, there is need for tests upon a large number of freshly isolated strains of the different pathogenic bacteria.

With regard to the substitution of liquorid-broth for tryptic-broth, though there is little demonstrable difference in the growth-promoting properties of the two, the less elaborate technique of preparation of the former renders it the more convenient routine medium.

### Summary

1 Sodium polyanethol sulphonate ("Liquoid") in concentrations of 0.03-0.05 per cent is effectively anticoagulant and destroys the bactericidal power of normal human blood for a large number of pathogenic bacteria. In concentrations of 0.1-0.2 per cent liquoid inhibits the growth of the strains of *N meningitidis* tested.

2 Liquoid-blood, without the addition of other culture fluids, is a suitable culture medium for the pathogenic bacteria tested.

3. Liquoid-broth is easily prepared, is unaltered by autoclaving, and keeps well. *In vitro* tests with artificially infected normal human blood suggest that the medium is worthy of trial in routine blood cultures.

We are indebted to the scientific department of Messrs Hoffmann-La Roche for the specimen of liquoid used in this investigation.

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# SOME SOURCES OF ERROR IN THE INTERPRETATION OF FERMENTATION REACTIONS, WITH SPECIAL REFERENCE TO THE EFFECTS OF SERUM ENZYMES

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FOR most fermentation tests peptone water has proved a satisfactory base for the medium, but there are many organisms, *e.g.* *N gonorrhææ*, which will not grow in it. With such fastidious organisms it is not unusual to add 1 to 10 per cent of normal horse serum to ensure growth. Thus it is not an uncommon practice to add horse serum to the fermentation media when examining the reactions of suspected diphtheria bacilli, a practice which does not appear to have led to wrong results with the two sugars most often used, glucose and sucrose. It is shown in this paper, however, that horse serum may invalidate tests with starch and maltose if it is added to the medium without previous inactivation of its enzymes. This difficulty was experienced in studies of the incidence and differentiation of the three types (*gravis*, *mitis* and intermediate) of *C diphtheriæ* (Anderson, Goldsworthy and Ward, 1936), of the possible differentiation of the equine and ovine types of *C ovis* (Carne, personal communication, 1936) and of the fermentation of maltose by various bacteria.

## *Fermentation of starch in the presence of normal serum*

**Experiments with *C. diphtheriæ*** In 1931 Anderson, Happold, McLeod and Thomson differentiated the *gravis* type of *C diphtheriæ* partly on the basis of its power to ferment starch. Since their report left some doubt as to the exact technique followed, various methods were tried in order to find a satisfactory way of testing the capacity of this organism to ferment starch. The first medium tried was that recommended by Andrews (1930) for the streptococcus—nutrient agar and 0.2 per cent of soluble starch (B D H). Since *C diphtheriæ* does not grow rapidly on unenriched nutrient agar, it was thought necessary to follow Andrews in adding approximately 1 per cent of horse serum sterilised by filtration

through a Seitz EK disc. On this medium the organism grew well and produced large colonies. After 24-48 hours' incubation the plates were flooded with weak iodine solution and examined for unstained haloes round the colonies. The differentiation of known *gravis* and *mitis* strains was wholly unsatisfactory as starch was apparently changed by both types, but a subsequent test showed that no starch could be detected by the iodine test even on an un inoculated plate.

Loeffler's coagulated serum containing 0.2 per cent. starch was also tested, but this too was quite useless, since the uninoculated and unincubated medium showed by the iodine test that all the starch had already disappeared.

In ordinary peptone water containing Andrade's indicator with the customary small inoculum growth was very slow. In order to increase the rate of growth, approximately 10 per cent of normal horse serum, filtered and unheated, was added, with the result that growth was enhanced, but again the *gravis* and *mitis* types could not be differentiated as both produced acid.

These results suggested that normal horse serum might either contain some substance fermentable by all types of *C. diphtheriae* (Andrewes, p. 149) or might act upon the starch so as to convert it into some fermentable substance. Experiment showed that the amount of fermentable carbohydrate in horse serum (measured as reducing substances\*) was not enough to produce an acid reaction when *C. diphtheriae* was grown in peptone water containing various concentrations of horse serum. A similar negative result was obtained by Spray and Doyle (1921).

To determine whether a hydrolytic enzyme was present various concentrations of normal horse serum were mixed aseptically with different quantities of starch and the mixtures incubated at 37° C. After 24-48 hours the iodine test revealed that certain concentrations of starch disappeared under the action of the serum, even of as little as 0.5 per cent. This test was performed by removing aseptically a large loopful of the mixture and adding to it on a spotting tile a similar loopful of weak iodine solution. It is thus easy to understand why the *mitis* strains appeared to be able to ferment the starch when normal horse serum was present.

Six strains of *C. diphtheriae*, three of *mitis* type and three of *gravis*, were grown in Hiss's serum water containing 0.1 per cent of starch. The medium was sterilised by autoclaving. They were also grown in 1 per cent peptone water containing a similar amount of starch and 10 per cent of unheated horse serum. After 24 and 48 hours' incubation both sets of cultures, together with un inoculated

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\* The total reducing substances as determined by the method of Hagedorn and Jensen amounted to 150 mg per 100 ml expressed as glucose. According to Campori (1935) the average figure for the horse is 50-100 mg per 100 ml.

controls, were examined for starch by the iodine test and for the development of an acid reaction to Andrade's indicator. In the heated medium, type differentiation was quite definite by both tests but rather less clear-cut with the iodine test. In the medium containing unheated horse serum the differentiation was completely masked, as acid was produced by both types of organism and the iodine reaction was negative in all inoculated tubes as well as in the controls. The amylase of normal horse serum may cause hydrolysis of starch but, if a completely transparent medium is not considered essential, autoclaved horse serum-water mixtures (or Hiss's serum water) form a satisfactory basis for fermentation tests.

**Experiments with *C. ovis*** One of us (J. A. D.), working with ovine and equine strains of *C. ovis*, had occasion to test their power to ferment starch. The strains were grown in

- (a) Hiss's serum water + litmus + 1 per cent starch,
- (b) peptone water + Andrade's indicator + 1 per cent starch,
- (c) peptone water + Andrade's indicator + 1 per cent starch + 10 per cent unheated horse serum.

Although it is generally accepted that *C. ovis* (ovine type) does not ferment starch, it was found that after 18 hours' incubation an acid reaction was present in all tubes containing normal serum. In Hiss's medium the equine strains produced an acid reaction but the ovine strains did not. In the peptone water medium growth was not appreciable. The serum of the ox, dog, cat, guinea-pig and hen behaved similarly to horse serum.

#### *Fermentation of starch in the presence of heated serum*

In a preliminary experiment one strain of *C. diphtheriae* (*mitis* type) was inoculated into 1 per cent peptone water containing starch and Andrade's indicator. Growth occurred in 22 hours but no acid developed in 96. With 5 per cent unheated horse serum growth was better and acid appeared in 22 hours. With the same concentration of serum heated at 100° C for one hour or 60° C for one hour no acid was formed.

Further experiments on similar lines with seven strains of *C. diphtheriae* (*mitis* type) entirely confirmed the conclusion that heating at 60° C for 60 minutes was adequate to destroy the amylase of horse serum. A more extensive experiment was then carried out with samples of serum heated for one hour at 45, 50, 55, 60, 65 and 70° C respectively, with identical results. When the starch peptone water contained 5 per cent of serum heated for 30 minutes at different temperatures and the completed media were kept at 37° C for several days before inoculation, it was found that the serum requires to be heated at 65° C for 30 minutes





of glucose, we found that heating the serum at 65° C for 60 minutes was necessary to destroy the maltase

## DISCUSSION

In 1846 Magendie described a "new" property of blood, namely, its ability to convert starch to sugar. Many years later Biel (1892) made a more detailed enquiry into the breakdown of starch by blood. He concluded that this action was due to a ferment, that the sugar produced was glucose and that the serum "diastase" was able to split maltose and achroo dextrin. In short he demonstrated that blood serum possesses both amylase and maltase. Subsequently (1892-93) he showed that human blood behaves in these respects like the blood of other animals but to a lesser degree. In the following year Tebb (1894) confirmed the presence of maltase in blood serum.

These well established facts were clearly recognised by Hiss (1901-05) when he devised the medium which now bears his name, but it is not at all certain that he fully realised the rapid action of such enzymes. He stated that heat at 65-70° C not only did not inactivate the "diastase" of starch serum mixtures, but even hastened its action. The apparent failure to obtain inactivation may well be attributed to the period of time elapsing between the mixing of the starch and serum and the attaining of a temperature of 65-70° C. It was this apparent failure to inactivate the serum at relatively moderate temperatures which led him to heat it at 100° C, a procedure which not infrequently produces an inconveniently opalescent medium. This opalescence makes it difficult at times to be sure that growth has taken place in the medium, so that the significance of a negative fermentation test is left in doubt.

In 1920 TenBroeck drew attention to the false results which might arise from the use of horse serum for the enrichment of fermentation media. He showed (p. 348) that the enzymes in horse serum, even after refrigeration for eighteen months, "will change maltose, dextrin and starch so that they will react as dextrose in media," and that these enzymes are destroyed by heating to 60° C for 15 minutes.

About the same time Spray and Doyle (1921) investigated the fermentation reactions of *S. pullorum* and *S. gallinarum* with special reference to maltose in an endeavour to differentiate them. In a Hiss's serum water maltose medium both organisms produced acid, whereas in infusion broth maltose medium *S. pullorum* did not. The contradictory results obtained with these two media were reproduced even when the media were sterilised for 15 minutes at 15 lb pressure. The Hiss's serum water maltose medium was prepared in the manner set out by Krumwiede, Kohn and Valcutino (1918), the serum, water, maltose and indicator being all mixed before any heat was applied. As mentioned in connection with Hiss's work it is conceivable that the serum enzymes produced their effect before the temperature of the steriliser had reached a level high enough to destroy them.

Somo years later Hendrickson (1926-27) attempted to confirm the suitability of maltose fermentation as a basis for differentiating *S. pullorum* and *S. gallinarum*. In order to ensure good growth of the organisms, he used a serum water medium. This was prepared and sterilised in the autoclave before the addition of any carbohydrate, thereby eliminating any action of the serum enzymes. He found that both organisms apparently fermented maltose in the serum water medium within 18 days. *S. pullorum* did not ferment maltose in the infusion broth medium until about the 40th day,

whereas *S. gallinarum* produced acid within the first few days. In view of the accepted ideas about the inactivation of serum enzymes it is inconceivable that the serum water medium prepared by Hendrickson should contain any active enzyme. Yet he reports that *S. pullorum* fermented the maltose in this serum water medium very much more rapidly than in infusion broth medium (1st day and 40th day respectively). The only explanation we are able to suggest is that the maltose was somewhat hydrolysed by exposure to a high temperature in the autoclave.

Hynd and Macfarlane (1927) examined the maltose-splitting activity of the serum of a number of animal species—pig, mouse, rat, guinea-pig, rabbit, kitten, ox and sheep. They found that pig serum alone had this power, a result which is in marked contrast to that obtained by one of us (J. A. D.), who found that horse, ox, dog, cat, guinea-pig and hen serum all rendered impossible the differentiation of ovine and equine strains of *C. ovis* on the basis of maltose fermentation. Glock (1936b) does not entirely agree with either of these views.

Pacheco and Rodrigues (1936) have further investigated the use of maltose for the differentiation of *S. pullorum* and *S. gallinarum*. They confirmed the presence in horse serum of a maltase-like substance which was not injured by exposure of the serum to a temperature of 60° C (no time given). They followed the action of serum heated at various temperatures upon maltose by means of a polarimetric method. In the absence of horse serum the fermentation of maltose offers a reliable means of differentiating the two organisms in question. These authors have discussed the same question in another paper (Rodrigues and Pacheco, 1936) in which they showed that heating at 60° C for 60 minutes was insufficient to eliminate the action of horse serum upon maltose.

Quite recently Murray (1935), in studying the stability of the *gravis*, *mitis* and intermediate types of *C. diphtheriae*, noticed that the *mitis* type when grown in broth containing serum appeared to acquire, transiently at least, the power to ferment starch. Even 0.1 per cent serum broth was able to induce this apparent change in the organism. Heating at 56° C for 30 minutes did not affect the activity of the rabbit serum. Other normal sera (ox, man, sheep and horse) were inactive, while guinea-pig serum resembled rabbit serum. The fact that this apparent change in the fermentative activities of the *mitis* type was evident only in the first subculture suggests that it was due to some factor carried over from the serum broth to the starch medium and it is tempting to regard Murray's results as due to the action of blood amylase.

In view of the established facts referred to above, it might reasonably be supposed that bacteriologists would have more generally appreciated the dangers attending the use of fresh blood serum in fermentation reactions. Nevertheless, it is still a common practice to add a little unheated sterile serum when investigating the fermentation reactions of delicate bacteria. It was through following this practice that our attention was drawn to these dangers, which were also mentioned to us by H. D. Wright in 1935. The results of our investigations confirm the reports by other authors that sterile serum used in fermentation media without reference to its enzyme content can lead to serious error in the interpretation of fermentation reactions.

Several authors have suggested heating the serum at various

temperatures in order to inactivate its amylase and maltase. These temperatures range from 60 to 100° C (Hiss) or even over 100° C (Pacheco and Rodrigues, 1936). Our own experiments indicate plainly that heating at 60° C for one hour is insufficient, but that heating at 65° C for 30 minutes is in general adequate to inactivate the enzymes responsible for the break-down of starch, glycogen and maltose. Since, however, exposing the serum to this temperature for more than 30 minutes in no way alters the transparency of the completed medium (although the serum in bulk becomes slightly opalescent), we would recommend that for safety's sake the serum be heated at this temperature for at least one hour. Certain specimens of horse serum, for reasons which we have been unable to determine, but which may be connected with the presence of haemoglobin, have become viscous or semisolid when heated to this extent.

Another source of error in the interpretation of fermentation tests carried out by the indicator technique is the more or less rapid reversion of the reaction of the medium to alkalinity. This reversion was particularly noticeable with certain *gravis* strains of *C. diphtheriae* in starch peptone water (Andrade's indicator). In this medium the degree of acidity as judged by the intensity of the colour was frequently very small and in some cases almost negligible. Practically every *gravis* strain tested was found to induce a complete reversion of the reaction within the second 24 hours of incubation, so that if the tubes were not inspected between the 15th and 24th hour after inoculation, the result was apparently negative. Such transient reactions are obviously liable to be misleading.

Wedum (1936) showed that the production of acid from a given carbohydrate may be so scanty or slow as to be neutralised by the buffer substances in the medium or by concomitantly produced alkali, so that there is no obvious production of acid. Wedum and Golden (1937) further investigated this phenomenon and confirmed the previous contention that the indicator method may give misleading results, especially when the utilisation of the carbohydrate is slow. Brown (1921) realised the possibility of this masking of acid production and suggested the use of media with a low buffer index when the observation of acid production is the only end in view. He discusses the principles involved at considerable length.

The possibility that there are in serum thermostable factors affecting the breakdown of carbohydrates must not be overlooked. It has been suggested by Pacheco and Rodrigues (1936) that such a factor exists for maltose and Wright (personal communication, 1935) has noticed that starch may disappear in the presence of heated serum. There seems little doubt that the degradation of starch is a process of some complexity and not well understood. Thus Glock (1936a) found that starch disappears when heated along with calcium chloride and phosphate buffer. If phosphate

is the active substance, as Glock would seem to suggest, it may not be so strange that serum, even after autoclaving, should retain some activity towards starch

### Summary

1 Horse serum contains amylase and maltase, even after storage for many weeks at 4° C These enzymes can lead to false interpretations when the serum is added to fermentation media To avoid this difficulty, serum should be heated for 60 minutes at 65° C before being added to the medium

2 There is disagreement between the results of some authors who have investigated the enzyme content of the serum of various animal species

3 Certain factors other than enzymes which may influence the apparent result of a fermentation test are discussed

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B L

## THE FERMENTATION OF GLUCOSIDES BY STREPTOCOCCI

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In a previous paper (Wright, 1937) it was shown that iodoacetic acid inhibits the formation of acid from sucrose by suspensions of various bacteria but that no evidence could be obtained of the accumulation of reducing sugars. Yeast suspensions however continued to produce reducing sugars from sucrose and bacterial suspensions to hydrolyse urea in the presence of concentrations of iodoacetic acid much greater than were required to inhibit acid production. This was considered consistent with the view that sucrose might be fermented by bacteria without preliminary hydrolysis, but could not be regarded as proof in view of the relative lack of delicacy of Fehling's reagent as a test for reducing sugars. Meyer (1933) has shown that iodoacetic acid also inhibits the fermentation and hydrolysis of certain glucosides by streptococci but records no details in his paper. He concludes that this indicates that the mechanism of hydrolysis of these glucosides by bacteria differs from that of hydrolysis by emulsin which, like hydrolysis by yeast and by ptyalin, is not inhibited (Lundsgaard, 1930). Experiments recorded in this paper fully confirm Meyer's findings and suggest that conclusions as to the mechanism of the bacterial fermentation of disaccharides which are based upon the effect of iodoacetic acid thereon must be very guarded as this substance appears to inhibit hydrolysis as well as fermentation.

### *Methods*

Most of the experiments were carried out with a streptococcus (333), isolated from milk, which has the general characters of *Str. thermophilus*. A few confirmatory experiments were done with a strain of *Str. faecalis*. For ordinary fermentation tests 0.1 c.c. of a fully grown broth culture was inoculated into serum peptone water containing 0.5 per cent of the substances tested and Andrade's indicator, readings being made at intervals up to 14 days. For experiments with suspensions, 0.1 c.c. of a 1:100 dilution of a broth culture was inoculated into 80 c.c. of broth containing 0.5 per cent of the desired carbohydrate. After 16 hours' incubation at 37° C the culture was centrifuged at high speed, the supernatant fluid poured off and the deposit taken up in 4 c.c. of a diluting fluid containing 0.85 per cent NaCl.

and 0.15 per cent  $\text{Na}_2\text{HPO}_4$ , the reaction being adjusted to pH 7.4-7.6. Usually one flask was required for each tube in the experiment. Of this suspension 3 c.c. were added to 30 c.c. of the same diluting fluid containing 0.3-0.5 per cent of carbohydrate. The glucosides used were salicin, arbutin, æsculin and amygdalin and were obtained from G. T. Gurr or the British Drug Houses, Ltd. They were prepared as 3 or 5 per cent solutions in the diluting fluid and sterilised by autoclaving for 10 minutes at 10 lb pressure, as this degree of heating was found to be permissible. On the night before the experiment 3 c.c. of these solutions were added to 27 c.c. of diluting fluid in 6×1 in. test-tubes which were placed at 37° C. until the end of the experiment. Æsculin is relatively insoluble in the cold, a 5 per cent solution becoming practically solid when cooled to room temperature. However when the solid mass is heated in a water-bath at 100° C. it rapidly liquefies and 3 c.c. can be added to 27 c.c. of fluid to make a 0.5 per cent solution which remains stable at 37° C.

Iodoacetic acid was used as a 1 per cent solution in distilled water carefully neutralised. From this the appropriate dilutions were made in diluting fluid and 3 c.c. added to 27 c.c. of diluting fluid and glucoside.

The progress of the fermentation was followed by estimating the pH colorimetrically at intervals. As æsculin forms somewhat acid solutions the initial reaction of fluids containing this substance was generally in the neighbourhood of pH 6.7. That of the other solutions ranged from pH 7.1-7.3.

Tests for saligenin, the aglucone produced on hydrolysis of salicin, were made with Jorissen's reagent (Sherman and Gross, 1911). To 5 c.c. of fluid were added 4 drops of a fresh 10 per cent solution of  $\text{NaNO}_2$ , 4 drops of 50 per cent acetic acid and 1 drop of 1 per cent  $\text{CuSO}_4$ . A reddish colour develops somewhat slowly, the intensity depending on the concentration of saligenin present. For æsculetin, the aglucone of æsculin, the test consisted of the addition of 1 drop of 1 per cent  $\text{FeCl}_3$  to 1 c.c. of fluid, which produces a green colour immediately. For quinol, the aglucone of arbutin, 1 drop of 5 per cent phosphomolybdic acid was added to 5 c.c. of fluid. With quinol this produces an immediate blue colour. No simple chemical test appears to be available for the aglucone of amygdalin but the characteristic smell of bitter almonds is of help. None of the specimens of salicin, arbutin or æsculin were found to contain free aglucone but one specimen of amygdalin contained traces of glucose and had to be rejected.

### *Fermentation of the glucosides*

*Ordinary fermentation tests.* The streptococcus 333 was maintained in 10 per cent milk broth and when tested in the ordinary way fermented salicin in 42 hours, æsculin and arbutin rather more slowly (48-72 hours) and amygdalin usually in from 6 to 12 days. The precise time varied a little in different experiments but salicin was always fermented most easily and amygdalin least, arbutin and æsculin occupying intermediate but not constant positions.

When cultivated in a medium containing one of the glucosides this streptococcus developed increased capacity to ferment that glucoside and sometimes others. In one experiment it was incubated in salicin broth for 48 hours, by which time it had fully developed.

It was now found to ferment salicin and arbutin in 18 hours, æsculin in 42 hours and amygdalin in 6 days. Another culture obtained by growth in amygdalin broth for 6 days fermented all four glucosides in 18 hours. The enzymes for the glucosides are evidently adaptive (Karström, 1930) and adaptation to salicin has always been found to result also in adaptation to arbutin and vice versa, a point of some interest as the aglucones of these glucosides are chemically not dissimilar but are very different from those present in æsculin and amygdalin. On the other hand growth in the presence of æsculin and amygdalin results in adaptation to all four substances (table I).

TABLE I

*Fermentation reactions of different cultures of streptococcus 333*

Carbohydrate used in preparing culture	Time taken to produce fermentation of carbohydrate (hours)					
	Glucose	Sucrose	Salicin.	Arbutin	Æsculin	Amygdalin
Glucose	18	18	42	48	72	108
Sucrose	18	18	18	18	96	192
Salicin	18	18	18	18	68	168
Arbutin	18	18	18	18	68	180
Æsculin	18	18	18	18	18	18
Amygdalin	18	18	18	16	18	18

*Streptococcus faecalis* fermented all four glucosides in 18 hours regardless of the medium upon which it had been grown.

*Experiments with suspensions.* Suspensions of streptococcus 333 showed also considerable variations in fermentative power according to the medium upon which the organisms had been cultivated. In table II are recorded representative results from experiments carried out over several months. The strains used

TABLE II

*Fermentation by suspensions of streptococcus 333 cultivated on different substrates*

Carbohydrate used in preparing suspension	pH attained in fermentation test with suspension.					
	Glucose	Sucrose	Salicin.	Arbutin	Æsculin.	Amygdalin.
Glucose	4.7	4.7	7.3	7.3	6.7	7.3
Sucrose	4.7	4.5	7.3	7.3	6.7	7.3
Salicin	4.6	4.7	4.7	5.7	6.7	7.3
Arbutin	4.7	4.7	4.9	4.6	6.7	7.3
Æsculin	4.9	4.9	4.9	4.6	4.9	5.1
Amygdalin	4.7	4.7	4.9	4.6	5.1	4.7

Initial pH of all tubes except those containing æsculin = 7.3

" " æsculin tubes = 6.7



were all derived from a milk broth culture by cultivation in broth containing one or other of the carbohydrates named until a heavy growth had developed, after which they were maintained in broth containing the same carbohydrate and this was also used in the preparation of the suspensions. Results have varied quantitatively from time to time but there has been no evidence that prolongation of the adaptation has significantly affected them. Such variations as have been observed have seemed to depend on the condition and strength of the particular suspension used.

The general result was in accordance with those obtained with the ordinary fermentation tests. It seems clear that the capacity to ferment salicin and arbutin is enhanced by growth in either of these substances but not that for æsculin or amygdalin, whereas cultivation in æsculin or amygdalin increases the activity for all four glucosides though the rate for salicin and arbutin is somewhat faster.

A strain which has been cultivated in amygdalin broth ferments all four glucosides in 18 hours in the ordinary test and if subcultured on glucose broth retains this power. Experiments with suspensions indicate that after a single subculture in glucose broth the strain is not so active as if maintained in amygdalin or æsculin (table III). Similar results were obtained with the other glucosides and with a strain which had been maintained in amygdalin for three months.

TABLE III

*Effect of cultivation in glucose after adaptation to glucosides*

Strain adapted to	Suspension prepared in	pH attained in 1½ hours in fermentation of					
		glucose	sucrose	salicin	arbutin	æsculin	amygdalin
æsculin	æsculin	4.7	4.7	4.7	4.7	4.9	6.4
„	glucose	4.7	4.7	7.3	7.3	6.7*	7.3
amygdalin	amygdalin	4.7	4.7	4.9	4.9	5.5	5.3
„	glucose	4.5	4.5	7.3	7.3	6.7	7.3

\* Initial pH of æsculin mixture = 6.7, of others = 7.3

In some experiments with suspensions grown in glucoside broth the fermentation of glucose appeared to be somewhat slower than that with suspensions from glucose broth. This did not appear to be an indication of loss by adaptation but seemed to depend on the relative density of the suspensions and other extraneous factors.

With *Str. faecalis* similar results were obtained. In the ordinary fermentation test all four glucosides were fermented in 18 hours but suspensions of culture from glucose broth were relatively inactive and adaptation enhanced their power.



TABLE IV.

*Effect of iodoacetic acid on fermentation and hydrolysis of salicin.*

Concentration of iodoacetic acid	1 1000	1 3000	1 10,000	1 30,000	1 100,000	Nil
Initial pH	7.3	7.3	7.3	7.3	7.3	7.3
pH after 3 hours	7.3	7.3	7.1	6.9	6.1	4.5
Test for saligenin at 3 hours	??	?	±	+	++	++++

Similar experiments were done with *æsculin* and *arbutin* with essentially the same results. Concentrations as low as 1 100,000 inhibited fermentation considerably and those round 1 10,000 prevented detectable alteration of pH. In the tubes containing no iodoacetic acid hydrolysis was active but it decreased in the presence of this substance. Usually a concentration of 1.1000 was required to abolish hydrolysis altogether. It is clear that there was no considerable accumulation of aglucone in one hour if fermentation was prevented and the results at three hours were sensibly the same.

An attempt was made to see if the concentration used considerably reduced the numbers of living organisms in the suspensions or affected them in any way so that their activity was reduced when the iodoacetic had been removed by centrifuging and washing. Reduction of the suspension to one-half by dilution had a marked effect on the rate of hydrolysis and fermentation of *amygdalin*, *æsculin* and *arbutin*. In one experiment with an *amygdalin* broth suspension the count of viable organisms at the beginning of the experiment was 310 million per c.c. The action of the suspension was tested on *arbutin* in the presence of iodoacetic acid, readings of fermentation and hydrolysis and counts of viable organisms being made after one hour (table V).

TABLE V.

*Effect of iodoacetic acid on viable count and fermentation.*

Concentrations of iodoacetic acid	1:1000	1 3000	1 10,000	1 30,000	1 100,000	Nil
Initial pH	7.3	7.3	7.3	7.3	7.3	7.3
pH at 1 hour	7.3	7.3	7.1	6.9	6.5	5.1
Test for quinol	..	---	+	++	++	+++
Initial count (millions per c.c.)	.	.	.	.	.	311
Count at 1 hour	178	196	220	271	299	300

In another experiment with the same strain it was found that dilution of the suspension did not completely abolish fermentation until the number of viable organisms was reduced to about

40 million per cc. It would seem that the effect of iodoacetic acid is not dependent upon this factor alone, for not even in the presence of the concentration 1:1000 is the number reduced by more than one half in one hour, though in three hours considerable reduction occurs.

Attempts to test the permanence of the effect of iodoacetic acid upon the streptococci were rendered difficult by the fact that repeated washing of suspensions reduces their activity. Virtanen (1926) has attributed this effect to removal of co-enzyme. We have found that a suspension which is washed 2-4 times in diluting fluid ferments sugars and glucosides more slowly than an unwashed suspension. If however 1 cc. of the supernatant fluid from a centrifuged glucose broth culture is added to 30 cc. of the test fluid the fermentation goes on at a much faster rate. Heating for 10 minutes at 10 lb. pressure does not destroy the effect of the supernatant fluid. Thirty cc. of streptococcal suspension prepared as described were divided into two equal parts. Half was mixed with 1:10000 iodoacetic acid in diluting fluid and half with diluting fluid only. Both preparations were kept at 37° C. for 30 minutes and then centrifuged and washed twice in diluting fluid warmed to 37° C. The activity of the suspensions upon amygdalin is shown in table VI.

TABLE VI

*Persistence of effect of iodoacetic acid on streptococcal suspensions*

Suspension	Quantity	Autolysed supernatant added	Initial pH	pH after 1 hour	pH after 4 hours
Treated with iodoacetic acid	3 cc.	Nil	7.3	7.3	6.7
" " "	3 cc.	1 cc.	7.2	7.0	6.1
Untreated " "	3 cc.	Nil	7.3	6.1	4.7
" "	3 cc.	1 cc.	7.2	5.3	4.5
" "	1 1/2 cc.	1 cc.	7.1	6.3	4.5

Several other experiments have given similar results and it seems clear that the effect of iodoacetic acid upon these streptococci is not so easily removed by washing as appears from Lundsgaard's work to be the case with yeast.

### Discussion

These experiments indicate that the enzymes in these streptococci for the glucosides salicin, arbutin, resacchin and amygdalin are adaptive in Karström's sense. The fermentation they produce is accompanied by hydrolysis which occurs very early and can often be detected at a time when no change in reaction of the fluid

has occurred, so that it would appear to precede or to occur simultaneously with fermentation. Hydrolysis of salicin and arbutin is apparently easier than that of æsculin and amygdalin, a point of difference from hydrolysis by emulsin and of resemblance to hydrolysis by acid. Adaptation of the enzymes to salicin and arbutin does not greatly influence their activity for æsculin or amygdalin but adaptation to either of the two latter results in increased activity for all four.

It was suggested in a previous paper (Wright, 1937) that failure to detect monosaccharides when sucrose was exposed to the action of various bacteria in the presence of iodoacetic acid tended to support the view that disaccharides might be fermented directly, without preliminary hydrolysis, especially as the bacterial hydrolysis of urea and hydrolysis of sucrose by yeast were not inhibited. The experiments reported in this paper seem however to weaken this particular argument. They confirm Meyer's findings that hydrolysis of these glucosides is inhibited by iodoacetic acid. It may be, as Meyer suggests, that the action of the bacterial enzymes differs from that of emulsin or it may be that the different result is due to a peculiar action of iodoacetic acid upon the bacteria. The inhibition does not appear to be wholly dependent on a reduction of the numbers of organisms in the suspension, but the peculiar persistence of the effect has been noted. It seems very unlikely that this type of substance should be directly fermented and as iodoacetic acid prevents the accumulation of the products of hydrolysis, apparently by inhibiting the hydrolysis itself, there is no reason why the action on the fermentation of disaccharides should not be of the same kind. Accordingly failure to detect monosaccharides in the experiments with sucrose cannot be held to prove that fermentation of sucrose is not preceded by hydrolysis. This conclusion in no way affects the arguments based on the study of a streptococcus with the peculiar ability to ferment certain disaccharides more rapidly than the constituent monosaccharides (Wright, 1936 *a* and *b*).

#### *Summary.*

- 1 The adaptive nature of the enzymes of two strains of streptococci for certain glucosides has been demonstrated
- 2 Fermentation of these glucosides is accompanied or preceded by hydrolysis
- 3 The ease of hydrolysis of the various glucosides is different from that observed with emulsin
- 4 Both hydrolysis and fermentation are prevented by iodoacetic acid
- 5 It is concluded that the absence of evidence of the accumulation of products of hydrolysis in the course of bacterial action in





# A CHRONIC AND FATAL FORM OF HYDROTHORAX PRODUCED IN RABBITS BY THE INTRAPERI- CARDIAL INJECTION OF TINCTURE OF IODINE AND THE MECHANISM OF ITS PRODUCTION

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(PLATES XVI AND XVII)

IN 1921 one of us (J H D), with G J Langley, carried out a number of experiments upon the effect of introducing a strong irritant—tinct iod fort (B P)—into the pericardial sac of rabbits. The intention was to set up a sterile adhesive pericarditis and to study the effects of the obliteration of the pericardial sac which should follow. The results of these experiments were surprising, in that although we produced the anticipated adhesions a very large number of animals died about 12½ days later with bilateral pleural effusion. A few animals survived for much longer periods and on being killed, although to all appearances healthy, showed pleural effusions.

The picture, when the chest was opened after death, was quite characteristic (fig 1). The two layers of the pericardium were adherent; sometimes the obliteration of the sac was complete, sometimes some hæmorrhagic fluid was present together with partial adhesion. Both lungs were collapsed and the pleural sacs full of clear straw-coloured effusion, which on each side might be as much as 30 cc. The fluid was rich in fibrinogen and clotted readily. Fibrinous pleural adhesions, fine and cobwebby in animals succumbing early but denser in those surviving longer, were often present. It was not usual to find fluid in the abdomen except as a terminal event. In most animals the liver was congested and in a few the organ showed fine cirrhotic scarring.

The present work was undertaken to clear up if possible the mechanism of these changes and to explain why the production of a sterile inflammation in the pericardial sac should result in a progressive bilateral hydrothorax.



*Technique.*

*The production of bilateral sterile hydrothorax* The normal method of introducing the tincture of iodine was as follows. The animals were given open ether anaesthesia and the skin over the sternum reflected by an incision an inch and a half long in the mid-line. The pectoral fascia and muscles were cleared from the ribs on the left side and the sternal ends of the 3rd, 4th and 5th ribs were defined. One blade of a blunt-pointed pair of scissors was now inserted into the 4th intercostal space and the 4th rib divided close to the sternal margin. This part of the procedure was a manoeuvre of some delicacy, for the internal mammary vessel lies close to the sternum and its division gives rise to annoying haemorrhage whilst, if the ribs were divided sufficiently far out to be certain of avoiding the vessels, a left-sided pneumothorax was produced which obviously was to be avoided in view of the nature of the experiments. The rib having been successfully cut a retractor was inserted and a view of the interior of the thorax could be obtained after the delicate fibres of the transversus thoracis muscle had been severed. In a successful exposure the heart could then be seen beating within the pericardium or, in adipose animals, this could be seen after the division of the substernal pad of fat which lies upon the anterior surface of the pericardium.

Once exposed, the pericardium was seized in fine rat-toothed forceps and about 0.2 c.c. of the material to be injected was introduced into the pericardial sac by means of a 10 c.c. syringe and a no. 17 hypodermic needle bent to a suitable angle. It was usually possible to satisfy oneself of the success of the procedure by seeing the injected material diffuse within the pericardial sac. No attempt was made to close the needle puncture in the pericardium or to suture the wound in the thoracic cage, but the pectoral muscles were stitched over the wound and the skin closed by a second continuous line of sutures, the wound being dressed with collodion and gauze. The fact that the muscles were divided in a different line to the skin aided the effective closure of the wound, which normally healed without sepsis.

This procedure was modified on occasion as circumstances demanded. Thus in a few instances the animals were given a preliminary intravenous injection of sodium hexyl-ethyl-barbiturate ("Hebaral," Parke, Davis & Co.), to reduce the time of ether inhalation where the pleural sac was deliberately opened.

*Controls.* Whilst no mechanical effects were anticipated from the small quantity of materials introduced, it was thought well to exclude the possibility of cardiac embarrassment from this cause and therefore in a number of control experiments equivalent quantities of sterile olive oil or liquid paraffin were injected. The results showed that such non-irritant substances, in the quantities used, did not produce any pericardial adhesions or hydrothorax and, further, that whilst some superficial sepsis occasionally complicated the healing of the skin incision the operative procedure did not set up any deep infection.

The remote possibility that the iodine itself might exert some specific effect apart from the irritative qualities of the tincture was also investigated, since it has been stated (Dixon, 1929; Chu and How, 1931; Sgalitzer, 1908) that sodium iodide is capable of producing pleural effusions in rabbits. Without going into details it may be said that the same results as those produced by the tincture of iodine could be obtained by the injection of 90 per cent. alcohol alone, and we are therefore justified in regarding the action of the tincture of iodine as a simple irritative one.

*The effects of the introduction of the irritant into other sites*

The effect of the injection of the same quantity of tincture of iodine into certain other situations was investigated, with the following results

(a) *Substernally* Here the injection was made into the tissues lying immediately anterior to the pericardial sac and in close relationship to it. The results, although a little less constant, were substantially the same as those in which the iodine was introduced intrapericardially, viz the production of pericardial adhesions, presumably from the diffusion of the irritant to the very closely related sac, and the ultimate supervention of hydrothorax. Exceptions mainly occurred in adipose animals in which there was a large pad of substernal fat. This presumably limited diffusion, absorbed a good deal of the irritant and protected the pericardium.

(b) *The root of the lung* Here the thorax was opened, the root of the lung exposed and the irritant dropped on to the structures forming its hilum. The result was negative. No hydrothorax was produced.

It may be mentioned that several of the animals which were subjected to this procedure died as soon as the tincture of iodine was placed in the desired position. Respirations became deep and irregular and finally ceased in full inspiration whilst the heart continued to beat for some minutes. The most probable explanation for this seemed to be direct stimulation of the vagus. Morrison (1920: 27) met with the same effect when he attempted to remove one lung in the rabbit by ligature of all the structures of the pulmonary root. It was found that the application of a local anaesthetic to the hilum area prior to the introduction of the iodine prevented these untoward results.

(c) *The pleural sac in the mid-axillary line* This situation was chosen as the one most remote from the pericardium and the root of the lung. The results here were also negative. A dense local fibrosis was frequently set up, which resulted in pleural adhesions from apex to base in the axillary region, but in no case was the mediastinal surface of the lung affected and no hydrothorax was ever produced.

The general outcome of these experiments was the conclusion that although it was not essential for the irritant to be introduced into the pericardial sac itself, the syndrome which we have described resulted from its action on the structures of the mediastinum most closely related to the pericardium. This syndrome could be produced with the greatest certainty by injecting the irritant intrapericardially and this technique was adhered to in our subsequent work.

## THE MECHANISM OF PRODUCTION OF THE HYDROTHORAX

A number of possibilities, some more likely than others, presented themselves for consideration. They may be taken *seriatim*.

## A. Congestive cardiac failure.

If the action of the irritant were limited to the heart and pericardium it might ultimately produce progressive cardiac failure from adherent pericarditis together with, perhaps, some myocardial fibrosis from the direct action of the irritant on the cardiac muscle. Although hydrothorax does not always accompany chronic cardiac failure in man, it is possible that it might do so in the rabbit. There are good reasons, however, for the rejection of this hypothesis for, although the degree of fibrosis which is produced in the parietal pleura following upon iodine injections is considerable, the hydrothoracic syndrome frequently made its appearance before a fibrotic reaction had time to develop and, moreover, with regard to the myocardium itself, histological examination of the heart in animals which had succumbed did not reveal any myocardial lesion.

## B. Local venous obstruction

This might arise from thrombosis, cicatricial constriction of the orifices of the pulmonary veins at their site of penetration of the pericardial sac, or by an inflammatory swelling of the tissues and lymph glands at the base of the heart. The latter tissues were always somewhat oedematous and, in the rabbit, the area included the pulmonary veins and superior vena cava.

All these possibilities have been investigated but no thromboses were ever found, the onset of the pleural effusions was early and established before any detectable fibrosis had developed, there was an entire absence of oedema or congestion of the head and upper limbs which should have resulted if the superior vena cava had been obstructed and, finally, animals which had received intrapericardial injections of iodine and which were killed by chloroform three days later often showed appreciable pleural effusion without noticeable enlargement of the mediastinal lymphatic glands.

There is no general agreement as to the exact relationship between venous obstruction and the production of oedema and effusions. The matter has been investigated experimentally by methods which increase the general venous pressure (Cohnheim, 1889, Bolton, 1903-04) and by those in which partial or complete occlusion of a large venous channel such as the inferior vena cava (Bolton, 1909-10) is produced. It may be recalled that Lower in 1669 claimed to have produced such "back pressure" oedema by the ligation of veins but this was disputed by Cohnheim. Moreover

Ranvier, who repeated Lower's experiments, showed that ligation of the inferior vena cava did not produce edema unless the sciatic nerves were also divided. More recent work by McMaster and Hudack (1932), Hudack and McMaster (1932) and Field, Drinker and White (1932) has indicated that, although increased venous pressure will not, *per se*, produce edema, there is nevertheless an increased production of lymph. The various views upon the formation of lymph in venous stasis are the secretory theory of Heidenham and Hamburger, the view that the accumulation of waste products influences the permeability of the capillary wall, allowing greater transudation of fluid, and the mechanical-hydrostatic theory which attributes the increased transudation mainly to increased venous and capillary pressure, with or without alteration in the semipermeable capillary membrane from nutritional changes.

A good deal of attention has been paid to these mechanical factors. Bolton (1909-10) produced a general increase in venous pressure by an elastic ligature which constricted but did not occlude the venous entrances to the heart and found that edema of the mediastinum and hydrothorax resulted. It is of interest for our present purpose that, although the venous pressure rose immediately the obstruction was set up, the rise was not maintained, yet where ascites had been produced by obstructing the inferior vena cava the fluid persisted in spite of the return of the venous pressure to normal, only disappearing when the lymphatic circulation was relieved of obstruction. Bolton's experiments show the lack of dependence of ascites on venous blood pressure and the difficulty of explaining it upon purely mechanical grounds. It is none the less clear that capillary pressure is a factor which must in many cases play a part and we would recall the experiments of Tainter and Hanzlik (1924-25) who showed that the edema produced by paraphenylenediamine could be prevented by lowering the systemic venous pressure.

The pulmonary vessels traverse the pericardium and receive a considerable serous investment from it. If these trunks were obstructed by the inflammatory reaction set up by the intrapericardial injection of iodine, visible and histological evidence of this should be forthcoming and some degree of pulmonary edema might be expected. That such obstruction would be of importance in producing hydrothorax is emphasized by the anatomical observations of Miller (1907-08) who showed that in man the venous blood from the visceral pleura passes into the pulmonary veins by intermediate capillaries, although the arrangement may be different in rabbits.

The condition of the pulmonary veins in a number of the animals succumbing to the cardio-hydrothoracic syndrome was examined by removing the parts entirely and fixing in 10 per cent formalin.

Dissections and histological sections at the level of the pulmonary veins, including the cardio-venous junctions, did not show any evidence of constriction by fibrosis or other mechanism.

*The azygos vessels.* When hydrothorax is present in cardiac failure in man it is found more often on the right side than on the left and, if unilateral, it is said to occur earlier and to be greater in quantity on the right side.

Thus Steele (1904) found the effusion to be greater in amount on the right side in three-quarters of his seventy-five patients, and of 13 cases of unilateral effusion 10 were right-sided. Further it is stated by Flint and da Costa (quoted by Landis, 1924) that the occurrence of hydrothorax bears no relationship to the severity of the cardiac lesion. To account for these anomalies it has been suggested that the all-important factor is the involvement of the larger azygos vein. It has been supposed that the downward traction of an enlarged heart might drag upon the vein as it loops over the root of the right lung and in this way obstruct the return of its venous blood, whilst Steele suggested that an enlarged right heart, and especially an engorged and dilated right auricle, might compress the vein and set up chronic passive congestion with resulting nutritional alterations in the capillaries and venules in its drainage area, thus producing right-sided hydrothorax. Fetterolf and Landis (1909) in opposing this view point out that the *vena azygos major* only drains a small part of the pleura, and that wholly parietal, whilst its collateral venous connections, especially its anastomoses with the azygos of the opposite side and with the lumbar veins, are extensive and free. The largest tributary of the azygos major is the azygos minor and therefore if compression of the larger vein were a factor in causing hydrothorax the condition should be bilateral. These workers stress the importance of obstruction to the pulmonary vein.

It is difficult to concede that the effusion in hydrothorax can arise solely or even chiefly from the parietal pleura, as the azygos hypothesis would require. Nevertheless we have thought it well to investigate the effect of occluding this vein experimentally.

### *The effects of ligature of the azygos vein*

Experiments were carried out to test the effect of this, since the iodine injections might cause fibrosis about the vein, or its thrombosis. Whilst the relative sizes of the two azygos veins in the rabbit do not differ as much as they do in man in many cases the right vessel was obviously the more important for the drainage of the parietal pleura.

The chest was opened on the right side by cutting two ribs in the mid-axillary line. The lung was collapsed and the animal very rapidly adjusted to the new conditions. A small self-retaining retractor was used to open the chest and a very fair exposure of the right azygos vein could be obtained by gently pushing the upper lobe of the lung to the side. The loop of the azygos vein lies alongside the vertebral column, just above the root of the lung, and can usually be seen except when the

A 15 cm. long piece of silk was used, with a blunt point ground at



blood stream by the simple expedient of heating the skin, and later, by micro dissection methods, it was shown by Landis (1927) that the damage done to the capillary endothelium by the pressure of a glass rod, although insufficient to allow indian ink particles to pass, permitted the escape of dyes (toluidine blue). He further noted that formaldehyde, ether, chloroform, and alcohol in sufficient strength produce similar results, and that the filtration of plasma proteins from capillaries damaged by 10 per cent alcohol in Ringer's solution occurs approximately seven times more rapidly than from normal capillaries.

Much detailed information regarding the nature of the process of dye leakage from damaged capillary endothelium is provided by Menkin and Menkin (1930). They used a colorimetric method of measuring the concentration of dye in capillaries and showed that the fall of concentration is twice as rapid in inflamed capillaries as in normal ones, a finding which they attributed to an increased permeability of the vessel wall produced by inflammation.

We have studied the permeability of the blood capillaries in the relevant areas in our rabbits which received intrapericardial injections of tincture of iodine. At different times after the operation a 1 per cent solution of dye in physiological saline was injected intravenously and the animals were killed at various intervals, the distribution of the dye being noted. Two typical experiments are described.

**Rabbit no 61.** Mid-sternal incision. Pectoral muscles divided and 4th rib cut at costo-sternal junction. 0.2 c.c. tinct. iod. fort. introduced into pericardial sac. No pneumothorax. Closure in two layers. Recovered well from anaesthetic and 20 hours later 4 c.c. of 1 per cent trypan blue introduced into marginal vein of ear. Twenty-four hours after the iodine injection the animal was killed by chloroform.

*Post-mortem.* The pleural sacs each contained about 10 c.c. of clear fluid, tinted blue. The pericardial sac contained blue fluid but not in excessive quantity. At the base of the heart there was what was taken to be an extremely large lymphatic vessel full of blue fluid and numerous smaller ones were seen coursing in the substernal fat. The basal lymph glands, prominent but not enlarged, were stained by dye. The bulk of the blue colouration was in the substernal fat and surrounding inflammatory effusion. The intercostal pleura was barely tinted. The peritoneum contained no free fluid and was not pigmented except for slight blue colouration in the intestinal wall. The receptaculum chyli contained pale blue lymph. A gland from the cardiac region of the stomach, which is constant, also appeared deep blue, as did a lymph vessel passing from it to the main ducts. The abdominal wall was normal.

**Rabbit no 59.** The same surgical procedure as in no 61. Eleven days later 5 c.c. of 1 per cent trypan blue was injected into the marginal vein of the ear. 24 hours later the animal was killed by chloroform.

*Post-mortem.* A considerable collection of blue pleural fluid (25 c.c. on each side). Apparently dilated lymphatics were seen in the sub-sternal fat. The lymphatics were well marked on the thoracic aspect of diaphragm, they mostly collected to a point situated sub-sternally, but others passed centrally to the interpleural membrane which divides the pleural sacs. Considerable oedema of abdominal wall. This was slightly tinged with blue. The bulk of the dye was concentrated in the mediastinum.





of Higgins' "white label indian ink" in physiological saline. This was preferred to other substances, such as colloidal silver, since it could be readily identified in histological sections of the regional lymph glands.

The animals were anaesthetised by the intravenous injection of sodium hexyl-ethyl-barbiturate in the dose of 80 mg. per kg. of body weight. The intrapleural injection was made with a needle attached to a rubber tube which led to a three-way tap, the other limbs of which were connected to a small water manometer and record syringe. The injection needle was pushed in a tangential direction into the chest, in or about the 4th intercostal space near the sternum, the manometer being carefully watched. As soon as a negative pressure was recorded, and the manometer fluid was swinging regularly with the respiration of the animal, the injection was made. Usually about 2.0-3.0 c.c. of the ink suspension were used. The animals were kept for varying periods and were eventually killed by chloroform.

*Post mortem* the lungs were carefully examined for puncture wounds and any animals showing these were excluded. The ink was usually found distributed in the pleural sac in masses, apparently bound up in fibrinous adhesions. It passed rapidly to the lymph glands, in which it was detectable in 24-48 hours. Naked-eye examination of these glands sufficed to show whether pigment was present, but in all cases a histological examination was also made, after fixation in 10 per cent formalin.

*Results* In all cases the pigment reached both sets of paratracheal glands, although the deep set received more than the other. The ink did not, however, reach the superficial basal cardiac glands unless some of it had leaked into the tissues of the thoracic wall. We therefore concluded that this group was not concerned with the drainage of the pleura and received its lymph mainly from the ventral portion of the thorax.

#### *The lymphatic drainage of the normal pericardium. .*

Similar experiments were carried out upon the lymphatic drainage of this sac. These were very disappointing. No ink appeared to leave the pericardium or reach the related glands and it was only after several weeks had elapsed that minute quantities of pigment might be found in the superficial basal cardiac glands and superficial paratracheal glands. Since it was impossible to prevent slight leakage of ink from the site of injection into the superficial tissues it seemed to us that this accounted for the small quantity of pigment found in the glands. Within the sac itself the injected ink was found at post-mortem to be distributed especially in the auriculo-ventricular sulci and about the line of reflection of the visceral and parietal pericardium.

*The investigation of normal animals therefore led us to the conclusion that whilst lymphatic absorption from the pleural sacs is abundant and rapid, no such absorption occurs from the pericardial sac or, at best, it is sparse and slow*

The latter observation confirms our earlier findings of 1921, and agrees with those of Shore (1927-28), who used carmine, and Drinker and Field (1931), who used methylene blue

*The lymph drainage in animals developing the hydrothoracic syndrome*

*The lymph glands* The functional condition of the thoracic glands was investigated in the animals with hydrothorax by following the distribution of particulate matter introduced into the effusion. Trypan blue and colloidal silver were tried but found too difficult to follow, so indian ink was used as in our experiments on the normal drainage. The ink was introduced by the manometric method already described in those animals in which an appreciable pleural effusion had not had time to develop, and by aspiration of some of the effusion and its replacement by a similar volume of ink in those animals which had a detectable hydrothorax. Although ink introduced into the pleural sacs will normally find its way into the glands with great rapidity we allowed as much time as possible to elapse before killing the animals so that ample time would be available for its passage if the pathways to the glands should be partially obstructed. Details of these experiments are given in the table

*Experiments on lymph drainage from pleura following iodine injection*

Expt no	Interval between iodine and ink.	Quantity of effusion withdrawn	Quantity of ink introduced (2 per cent. suspension)	Interval between ink and death	Presence of ink on microscopic examination of lymph glands		
					Superficial basal cardiac group	Superficial paratracheal group	Deep paratracheal group
R 37	5 days	3 cc right chest	50 cc	2 days	—	Few small granules in macrophages in medulla doubtful if ink	—
R 41	9 "	3 cc right	15 cc	4 "	—	—	—
R 42	9 "	3 cc "	10 cc	4 "	—	—	—
R 44	16 "	15 cc "	20 cc	24 hrs	—	—	—
R 44a	9 "	5 cc "	10 cc	24 "	—	—	—
R 63	Simultaneously	None	40 cc	0 days	—	—	—
R 65	24 hrs	3 cc right	into left pleura 40 cc	4 "	—	—	Trace on right side*
R 67	24 "	None	30 cc	2 "	—	—	—

\* There was less effusion than one would have expected after 5 days and the animal appeared largely to have recovered from the iodine

In all of these experiments the naked-eye observations were confirmed by microscopical sections of the mediastinum at different levels and only in the two instances indicated in the table was a minute quantity of pigment found in the glands

The histological appearances are illustrated by figs 2-5, which show glands of identical groups in control animals (figs 2 and 3) and in those with the hydrothoracic syndrome (figs 4 and 5) The stuffing of the glands with pigment in the first and its absence in the second are well shown Details of three typical experiments follow

**Rabbit no. 42** Small black and white male Ether anaesthesia Procedure as usual for intrapericardial iodine injection Very fair exposure but some difficulty in grasping pericardium A mixture of 0.2 c.c. tinct iodine fort and 0.2 c.c. 10 per cent planocaine was used for injection, and 0.3 c.c. of this was introduced intrapericardially Closure in routine manner Recovery good Nine days later, 3 c.c. clear straw coloured fluid aspirated from right chest and replaced by 1 c.c. of 2 per cent indian ink in sterile normal saline Four days later the animal died

*Post-mortem* Large collections of pale translucent fluid in both pleural sacs Masses of flocculated ink lying in region of mediastinal pleura on right side, also towards lower lobe of right lung A few patches of pigment adherent to intercostal and diaphragmatic pleura easily brushed off surface There appeared to be some black lines coursing in the pleurae separating the two sides of the thoracic cavity (? lymphatic vessels) Traced downwards these led to a large mass of flocculated ink, whilst upwards they passed to the substernal fat where they appeared to end abruptly and could not be further identified The substernal group of lymph glands was prominent. Paratracheal groups on both sides appeared to be increased numerically rather than in size and completely embraced the great vessels at the base of the heart The groups of glands were dissected out and sections made after fixation in 10 per cent formalin No evidence of pigment in these glands (fig 4)

**Rabbit no 63** Small black and white male. Ether anaesthesia 0.2 c.c. tinct iodine fort introduced into pericardial sac by procedure of cutting 4th rib No haemorrhage, no pneumothorax Immediately afterwards 4 c.c. of 2 per cent sterile indian ink in normal saline were introduced into left pleural sac through incision made for exposure of heart Closure in two layers Recovery good Five days later, killed by intravenous injection of air.

*Post-mortem* Considerable bilateral pleural effusion, that on left stained by ink, which was also adherent to pleural surfaces in flakes. Transverse sections of mediastinum at level of paratracheal lymph glands made after fixing in 10 per cent formalin Pigment absent from these glands

**Rabbit no 67** Large grey male Ether anaesthesia Iodine injected into pericardial sac by routine method 0.2 c.c. tinct iodine fort introduced satisfactorily No haemorrhage, no pneumothorax Twenty-four hours later, 3 c.c. of 2 per cent sterile indian ink in normal saline introduced into right pleural sac by manometric method Two days later, killed by chloroform inhalation

*Post-mortem* Considerable effusion in each pleural sac Flocculated ink in right side There was also some ink in the pectoral muscle, indicating that the needle cannot have been in the pleural space during the whole period of injection Fixation of heart and lungs in 10 per cent formalin



## PLATE XVI

- FIG 1 —General condition in the thorax of a rabbit about two weeks after intrapericardial injection of iodine. The left pleural sac has been emptied, exposing the collapsed lung. On the right side can be seen the fine adhesions on the pulmonary surface.
- FIG 2 —Section of paratracheal lymph gland of rabbit no. 37a removed 2 days after introduction of indian ink into pleural sac. No intrapericardial iodine. Note the stuffing of the lymph paths with pigment.  $\times 36$
- FIG 3 —Section of paratracheal lymph gland of rabbit no. 56 removed 4 days after introduction of indian ink into pleural sac. No intrapericardial iodine. Note stuffing of lymph paths with pigment.  $\times 36$
- FIG 4 —Section of paratracheal lymph gland of rabbit no. 42 removed 4 days after introduction of indian ink into pleural sac. Intrapericardial iodine given 13 days previously. Note the complete absence of pigment.  $\times 37$
- FIG 5 —Section of paratracheal lymph gland of rabbit no. 67 removed 2 days after introduction of indian ink into pleural sac. Intrapericardial iodine given 24 hours previous to ink. Note the complete absence of pigment.  $\times 37$

EXPERIMENTAL HYDROTHORAX



FIG 2

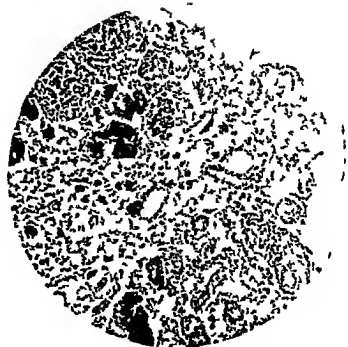


FIG 3



FIG 1



FIG 4

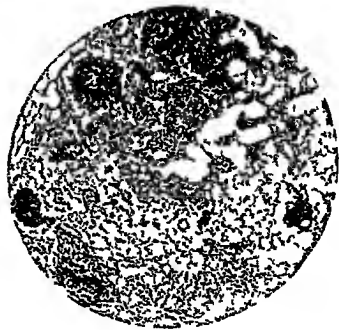


FIG 5



EXPERIMENTAL HYDROTHORAX



FIG 6—Dilated mediastinal lymphatic vessel containing fluid Rabbit no 44  $\times 44$

FIG 7—Dilated lymph vessels 13 days after intrapericardial iodine Rabbit no 41  $\times 38$



FIG 8—Dilated lymph vessel containing large numbers of lymphocytes removed 24 hours after infiltration of right and left lymphatic ducts with iodine Cf figs 6 and 7 Rabbit no 53  $\times 40$





Transverse sections at level of paratracheal and substernal lymph glands. Microscopic examination reveals no evidence of deposition of ink in the lymph glands (fig 5)

*These findings appear conclusive once the hydrothorax has been initiated there is a complete obstruction to the passage of ink to the lymphatic glands draining the pleural sacs*

Furthermore, where, in certain experiments, the injection of iodine into the pericardial sac was simultaneous with the introduction of ink into the pleura, it was found that no pigment reached the mediastinal glands. It is therefore clear that the interruption to the normal lymph flow occurs with considerable rapidity.

From this evidence we feel justified in concluding that in addition to an inflammatory exudate in the area affected by the irritant there is at the same time an obstruction to the lymphatic drainage. To some extent, and in the peculiar circumstances of our experiments, this would lend support to the views of Opie (1929) and Menkin (1931), both of whom maintain that in inflammatory areas absorption by the lymphatics is impaired. Menkin is convinced that the mechanism for this is the plugging of lymph vessels by fibrinous thrombi. We have searched many sections of the mediastina in our animals but in none have we found any evidence of thrombosis, though many patent lymph vessels were seen. Rather, on the other hand, were the vessels large and apparently full of liquid (figs 6 and 7), supporting the view that in oedematous states the lymph vessels have a greater calibre than normal instead of being compressed and small (Pullinger and Florey, 1935). Moreover the sections of the lymph glands suggest that they are the recipients of an increased quantity of fluid, since most of the sinuses are dilated.

Given, then, that some sort of obstruction is created which prevents the exuded pleural fluid from being reabsorbed by way of the lymphatic glands, it remains to ask at what site on the pathway the obstruction takes place. Unless we are to assume that the lymph circulation comes to a standstill the blockage may be at one of several points: (i) the serous surface itself, (ii) in the lymph vessels between the serous surface and the regional lymph glands, (iii) in the glands themselves or in their efferent vessels.

(i) For closure of entry to the lymphatics at the serous surface we must assume either a fibrinous exudate or a fibroblastic proliferation sufficient to produce such an effect. But fibrin itself forms a very permeable network which moreover did not usually, in our experiments, cover the whole of the pleural surface, whilst the fluid exudation into the pleural sac has been demonstrated as shortly as 24 hours after the intrapericardial injection and before any effective fibroblastic proliferation could have occurred. Since indian ink failed to reach the regional lymph glands from the

pleural sac when it was injected at the same time as the intrapericardial iodine injection, or 24 hours afterwards, the intervention of a fibrinous exudate would seem to be excluded and, *a fortiori*, that of a cellular reaction. We would also draw attention to the fact that in certain animals sections of the pleura on the ink-injected side showed that the pigment had penetrated beneath the serosa.

(ii) *The intermediate lymph vessels.* Obstruction to these in their course between the serous surface and the glands would appear to be the most probable event. Such a blockage might be due to greatly increased permeability of their walls as a direct effect of the diffusion of the iodine, allowing leakage of the transported lymph, it might be due to mechanical obstruction by thrombosis or endothelial proliferation and, finally, the viscosity of the pleural effusion might be incompatible with its rapid removal. Although no quantitative chemical estimations of the composition of the effusions have been made there is evidence of a high fibrinogen content and the effusion, when removed, rapidly formed a large and firm clot. The lymphatic vessels, which for the most part in the mediastinum, were repeatedly examined histologically, in normal animals which had received intrapleural injections of ink this could be readily seen within them but in the iodine-treated animals with hydrothorax no ink was present in this situation. There was likewise no evidence of pigment having entered and leaked from the vessels. The thrombi described by Menkin (1931) were never encountered, although the vessels which he found obstructed in this way were those at present under discussion.

(iii) *The lymphatic glands and their efferent vessels.* Since ink failed to reach the glands from the hydrothorax fluid it would seem that the obstruction as we have pointed out must be on the pleural side of the glands. It is just conceivable however that an obstruction to the efferent vessels would impair the flow of lymph into and through the glands from their drainage areas, so that the possibility of a proximally acting obstruction had to be considered. We have attempted to produce this condition and to compare the picture so produced with that in our animals with the hydrothoracic syndrome.

A preliminary investigation of the anatomy of the thoracic and right lymphatic ducts after the intraperitoneal introduction of 20 c.c. of 1 per cent potassium iodine a quarter of an hour before, showed that by incisions through the peritoneum 15 over the clavicle these ducts could be identified easily on each side by the position of the subclavian and jugular veins on either side.

For this purpose the animals were placed in the operation of exposure of the ducts, which was not too difficult for the purpose by feeds of milk an hour before the operation. The ducts were exposed by incision and introduced into the pleural cavity by means of the ducts with the veins in an attempt to produce a proximal obstruction in the lymph vessels. Two of the animals were killed at intervals of 24 hours, the other animals were killed

for 24 hours following the operation. The details of this procedure and the findings in one animal are given below; those in the second animal were in all essentials identical.

**Rabbit no 53** The operative procedure already described was carried out. The animal recovered immediately from the operation but died on the following day. The abdomen was distended. There was no oedema of the head or upper extremities. On opening the abdomen an excess of free fluid was found in the peritoneal sac, the intestines were dilated and their contents appeared unusually fluid. The lacteals were distended and readily traceable to Aselli's pancreas at the root of the mesentery. Some fibrinous deposits were present on the serosa of the stomach. Parasitic cysts were present in the liver and peritoneum. There was an excess of free fluid—about 30 cc—in each pleural sac. The lungs appeared emphysematous. The usual mediastinal glands were enlarged and milky white in colour and it was possible to see some of the efferent lymphatics as white knotted cords lying along the aorta. The pericardium contained an excess of free fluid. The heart was not enlarged.

Histological examination of the glands in these animals showed a condition (fig 8) which contrasted very markedly with that found in our previous experiments (figs 6 and 7). Both afferent and efferent lymphatics were full of lymphocytes and the glands themselves were packed with cells. The picture is one of lymph obstruction whereas that in the animals with the hydrothoracic syndrome was not, the glands in the latter having widely patent lymph corridors and sinuses and dilated efferent vessels containing an excess of oedema fluid. These experiments therefore give no support to the suggestion that the obstruction to fluid return from the thoracic cavity was located in the glands or their efferent vessels.

The sum of our results, then, indicates that whilst particulate matter in the hydrothorax fluid can make its way for a certain distance into the tissues surrounding the serous sacs it cannot—in sharp contrast to what pertains in the healthy animal—proceed further. What holds it up remains to some extent obscure. There is no evidence of lymphatic thrombi. The appearance of the glands does not help, for their dilated sinuses and efferent vessels would suggest that they are receiving an excess of fluid from local inflammatory changes set up by the irritant, rather than that they are involved in any obstructive process.

### SUMMARY

A form of bilateral chronic and fatal hydrothorax in rabbits is described which was produced by the intrapericardial injection of a strong irritant such as tinct iodine fort (B.P.). The investigation of the mode of production and maintenance of this fluid effusion showed that it could not be attributed to cardiac embarrassment from

changes produced either in the heart itself or in the pericardial sac, nor to any form of gross venous obstruction resulting from inflammatory or cicatricial changes

Out of the many possible factors investigated, two emerged which it is concluded are of chief importance. In the first place the permeability of the vessels which supplied fluid to the serous surface was shown by dye experiments to be increased, since trypan blue, a dye of large molecular weight, was capable of passing out into the effusions. Secondly the resorbing mechanism was obstructed. Particulate matter, which normally passed readily from the pleural sacs to certain of the mediastinal glands, now failed to do so although still capable of traversing the tissues for a certain distance. It seemed, in the absence of more direct evidence, that the movement of fluid in the inflamed area occasioned by the irritant, which lay in the route from the pleura to the lymphatic glands, was disturbed. No lymphatic thrombosis or gross cause of this disturbance was found but it is suggested that the high protein content and viscosity of the fluid exudate was such that the physical forces available were incapable of moving it across the inflammatory zone. We suggest that such qualitative disturbance in the tissue fluid in inflammation is a factor of importance, rather than the quantitative disturbance alone, in producing the tumor and oedema of inflamed tissues.

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# THE COMBINING POWER OF STAPHYLOCOCCUS TOXOID THE DANYSZ EFFECT AND OTHER FACTORS INFLUENCING ITS DETERMINATION

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It has long been known that antitoxin combines with more toxin if the toxin is added all at once than if the addition is made in two parts with a time interval between. This effect was first observed by Danyasz (1902) for ricin and for diphtheria toxin. Similar observations were made by Sachs (1904) for staphylolysin, and by Madsen and Arrhenius (1906) for tetanolyisin.

## DANYSZ EFFECT WITH STAPHYLOCOCCUS TOXIN-ANTITOXIN MIXTURES

We have confirmed and extended the experiments of Sachs on staphylococcus toxin-antitoxin combination and have demonstrated the Danyasz effect in a titration of staphylococcus a toxin with antitoxin by the rabbit cell hæmolytic method.

Two series of toxin antitoxin mixtures were set up. In both series, graded amounts of toxin were added to a constant amount of antitoxin, but in series 1 the toxin was added in one dose while in series 2 half the toxin was added initially and the second half 4 hours later. Both series of mixtures were allowed to combine for 30 minutes at room temperature after the last addition of toxin, a constant dose of washed rabbit erythrocytes was then added to each tube and the degree of hæmolysis was read after 60 minutes' incubation at 37° C.

It will be seen from table I that one unit of antitoxin will just neutralise 0.15 c.c. of the toxin when this is added in one portion, but that the same amount of antitoxin will neutralise only about 0.12 c.c. of toxin if it is added in two equal portions at an interval of 4 hours.

The degree of displacement depends on the proportion of the total toxin added initially. As this proportion decreases from 100 to 0 per cent, the total toxin required falls to a minimum and then rises again. The maximum displacement occurs where the



TABLE I

*Danysz effect in staphylococcus toxin-antitoxin combination*

Series 1. Toxin added all at once. Mixtures incubated $\frac{1}{2}$ hr before addition of cells.		Series 2. Toxin added in two portions with interval of 4 hrs Mixtures incubated $\frac{1}{2}$ hr after 2nd addition		
Dose of toxin (c c)	Hæmolysis	1st addition of toxin (c c)	2nd addition of toxin (c c)	Hæmolysis
0 008	—	0 049	0 049	—
0 108	—	0 054	0 054	—
0 12	—	0 06	0 06	tr
0 132	—	0 066	0 066	p
0 146	—	0 073	0 073	c
0 16	p	0 08	0 08	c
0 176	p	0 088	0 088	c
0 194	ac	0 097	0 097	c

All tubes in both series contained 1 unit of antitoxin and the total volume was the same. Toxin used no 28A.

c = complete hæmolysis.                      p = partial hæmolysis  
ac = almost complete hæmolysis              tr = trace hæmolysis  
— = no hæmolysis

first and second doses of toxin are about equal. This is illustrated by an experiment the results of which are recorded in table II.

Seven series of mixtures were set up. In each series graded amounts of toxin were added to one unit of antitoxin, the toxin being added in two fractions with a time interval of one hour. The proportion of toxin added initially to that added subsequently decreased from series 1 to series 7. The mixtures were allowed to stand on the bench for 30 minutes after the second addition of toxin, after which the red cells were added and the test was completed as before.

It will be seen that when the first and second doses of toxin are approximately equal (series 5) the shift in end-point is 18 per cent, whereas in series 2 in which the initial dose represents eighteen-nineteenths of the total toxin required the displacement is only

TABLE II

*Influence on the Danysz effect of varying the proportion between the first and second additions of toxin.*

Series no.	1st dose toxin (c c)	2nd dose toxin (c c)	Total toxin (c c)	Toxin required as fraction of 1st dose
1	0 16	0	0 16 (= 1h)	(1 00)
2	0 146	0 005	0 151	0 96
3	0 133	0 0165	0 150	0 91
4	0 110	0 043	0 143	0 89
5	0 066	0 066	0 131	0 82
6	0 016	0 129	0 145	0 91
7	0 008	0 157	0 165	1 00

Figures are in volumes of toxin (50 D) required to cause hæmolysis when added to 1 c c of antitoxin. Toxin was added in two fractions at an interval of 1 hour.

4 per cent, and in series 6 in which the initial dose is only one-ninth of the total toxin required the displacement is 9 per cent. Similar observations were made by von Dungern (1904) for diphtheria toxin, by Sachs (1904) for staphylolysin and by Madsen and Arrhenius (1906) for tetanolysin.

### *Reversal of the Danysz reaction*

Arrhenius (1907), reporting some experiments by Madsen, stated that the Danysz effect was not permanent. The abnormal toxicity of tetanolysin-antilysin mixtures produced by adding the toxin in fractions was slowly lost and after 6 hours at 37° C the mixtures were no more toxic than if the toxin had been added in a single dose.

In staphylococcus toxin-antitoxin titrations by the hæmolytic method it is our practice to record hæmolysis after incubation for one hour at 37° C and again after the mixtures have stood on the bench overnight. Hæmolysis progresses somewhat between the first and last readings but we have observed that the progression is less in those series in which the toxin has been added in two parts than in those in which a single addition of toxin has been made. In other words, the Danysz effect appears less pronounced at the overnight than at the earlier reading. This may be due to reversal of the Danysz reaction but such reversal is slow in staphylococcus toxin-antitoxin combination. If an interval of 24 hours be allowed to elapse between the second addition of toxin and the addition of red cells, the extra toxicity is less than if the interval is one hour, but it is not entirely suppressed.

### *The mechanism of the Danysz reaction*

Two hypotheses have been advanced to account for the Danysz phenomenon. von Dungern suggests that toxic filtrates contain two components, both of which combine with antitoxin, only one being toxic. He further supposes that the toxin combines more readily with antitoxin than the non-toxic factor which he calls epitoxonoid. The L+ mixture of toxin and antitoxin contains one lethal dose of toxin free and will, according to von Dungern, contain also considerable amounts of free epitoxonoid. Thus the measurement of the L+ dose will not give a measure of the total antigen present in the filtrate. von Dungern, however, postulates that epitoxonoid will combine completely with antitoxin if the latter is present in excess and that, once combined, it is only very slowly displaced from its combination by the addition of toxin. If, therefore, half the L+ dose of toxin is added to one unit of antitoxin all the antigen combines and will take up more than half a unit, so that less toxin than half the L+ dose is now necessary to render the mixture lethal. There seems insufficient reason for

distinguishing between von Dungern's epitoxonoid and the substance which is now called toxoid. His arguments would equally apply to the mixture of toxin and toxoid present in bacterial filtrates.

The second hypothesis is that toxin and toxoid can combine with antitoxin in more than one proportion. Healey and Pinfield (1935) claim to have shown that two compounds of diphtheria toxin and antitoxin exist,  $TA$  and  $TA_2$ . Toxin, or the neutral compound  $TA$ , will combine with excess antitoxin to form  $TA_2$ . On this hypothesis, when toxin is added to antitoxin in two fractions, the initial fraction combines with the excess antitoxin to form  $TA_2$ . When the second addition of toxin is made this antitoxin is not available for its neutralisation and a more toxic mixture results than would have occurred if the toxin had been added all at once. Healey and Pinfield showed that the compound  $TA_2$  is unstable in the presence of toxin and reacts with it relatively slowly to form  $TA$ .

#### *The significance of the flocculation reaction.*

According to von Dungern, the Danysz phenomenon is explained by the fact that toxin has a higher affinity than toxoid for antitoxin. As pointed out by Glenny (1931) a corollary of this hypothesis is that the Danysz effect should not be demonstrable in flocculation reactions, in which total antigen (toxin+toxoid) is measured. S. Schmidt (1927, 1928), however, was able to show that the "indicating tube" in flocculation tests with tetanus or diphtheria toxin and antitoxin was displaced towards the side of excess antitoxin when the toxin was added in two fractions.

We have not been able to confirm Schmidt's finding, with staphylococcus toxin and antitoxin. The mixture to flocculate first has exactly the same composition whether the toxin is added in a single dose or in two parts.

#### THE DETERMINATION OF THE COMBINING POWER OF TOXOID.

##### *The significance of the Danysz phenomenon*

*Néls's method* Owing to the fact that formalin destroys the hæmolytic action of staphylococcus toxin, estimations of the combining power of toxoid by the hæmolytic method involve the addition of an "indicating dose" of toxin to the toxoid-antitoxin mixtures. In the method described by Néls (1935) quantities of antigen in series, including the expected  $Lb^*$  dose, are each mixed with two units of antitoxin and the mixtures allowed to stand for 20 minutes, after which a full  $Lh$  dose of toxin (previously ascertained) is added as the indicating dose and the mixtures are again left for 20 minutes before the addition of red cells.

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\* The  $Lb$  dose of a toxoid is the dose which just binds one unit of antitoxin.

In making some comparisons of the potency of samples of staphylococcus toxin and of their formol-toxoids it was found that the power of combining with antitoxin had apparently increased during formolisation. For the toxin samples the reciprocal of the Lh dose was taken as a measure of combining power, for the toxoids the reciprocal of the Lb dose as determined by the method of Nélis. The apparent increase in combining power is explained by the fact that the estimations of toxin and toxoid were not carried out by comparable methods. In determining the Lh dose of the toxin the antigen was added all at once, whereas in the estimation of the Lb dose of the toxoid the addition of antigen was in two parts. Whatever the cause of the Danysz phenomenon it is clear that it should be taken into consideration when such comparisons are made. If both toxin and toxoid are titrated by the method of Nélis, then, since both antigens have been added fractionally to the antitoxin, a Danysz reaction occurs in both instances and, provided the tests are performed under the same conditions and the same sample of toxin is used as indicator, the reciprocals of the Lb doses observed will give comparable measures of the relative potencies of the toxin and of its formol-toxoid. Table III shows such an experiment in which the combining power of toxoid 37A has been estimated by the method of Nélis and that of the toxin of origin both by the method of Nélis and by the Lh method.

Table III shows that if the potencies of toxin and toxoid are compared by the same method the combining power of the toxoid appears the lower, whereas if the Lb dose of the toxoid is compared with the Lh dose of the toxin it would appear as if formolisation had increased the combining power.

Owing to the fact that different samples of toxin contain different proportions of toxoid, estimates of the potencies of antigens may not be strictly comparable unless the same indicating toxin is used in all tests. It should also be borne in mind that if much time is allowed to elapse between the addition of toxin and the addition of red cells, reversal of the Danysz reaction may have set in. Since for staphylococcus toxin and antitoxin this reverse reaction is very slow, the deviation due to this cause should not be great.

*McClellan's method* Another method for comparing the combining powers of toxins and of the toxoids derived from them under comparable conditions is that advocated by McClellan (personal communication). In McClellan's method the toxoid is blended with an equal volume of toxin and the Lh dose of the blend is determined in the ordinary way. Since the antigen is added all at once the Danysz phenomenon does not affect this method. The combining power of the toxoid is derived by calculation from a knowledge

of the Lh doses of the indicating toxin and of the toxin-toxoid blend. An example is given to show the method of calculation

Lh dose of indicating toxin  $= 0.14 \text{ c.c.}$   
 Lh dose of mixture of equal parts toxin and toxoid  $= 0.17 \text{ c.c.}$   
 0.17 c.c. blend contains 0.085 c.c. toxin + 0.085 c.c. toxoid,  
 so that 0.085 c.c. toxoid is equivalent to  $0.14 - 0.085 \text{ c.c.}$   
 toxin  $= 0.055 \text{ c.c.}$   
 toxin  
 The Lb dose toxoid will thus be  $\frac{0.085 \times 0.14 \text{ c.c.}}{0.055} = 0.22 \text{ c.c.}$

If the toxin of origin is used as indicating toxin, the ratio of the combining power of the formol-toxoid to that of the toxin of origin becomes  $\frac{0.055}{0.085}$  or 65 per cent.

TABLE III.

*Influence of method on the comparative estimations of combining power before and after formolisation*

Series 1 Combining power of toxoid 37A by method of Néls					
Dose of toxoid (c.c.)	0.069	0.083	0.10	0.12	0.145
Dose of antitoxin (units)	2	2	2	2	2
Dose of toxin 37A (c.c.)	0.14	0.14	0.14	0.14	0.14
Hæmolysis	—	—	—	p	c
Lb of toxoid taken as 0.12 c.c.					
Series 2 Combining power of toxin 37A by Lh method					
Dose of toxin (c.c.)	0.098	0.117	0.14	0.168	0.20
Dose of antitoxin (units)	1	1	1	1	1
Hæmolysis	—	—	p	ac	c
Lh taken as 0.14 c.c.					
Series 3 Combining power of toxin 37A by method of Néls					
Initial dose of toxin (c.c.)	0.068	0.081	0.097	0.116	0.139
Dose of antitoxin (units)	2	2	2	2	2
2nd addition of toxin (c.c.)	0.14	0.14	0.14	0.14	0.14
Hæmolysis	—	—	p	c	c
Lb of toxin taken as 0.097 c.c.					

One advantage of McClean's method is that if the toxin of origin is used as indicator and if the toxin-toxoid blend is graded from the Lh value of the toxin to double that value, the hæmolytic end-point will always be on the scale even if the toxoid under test has no combining power left. The disadvantage of the method

lies in its lack of precision. In this method the precision of the calculated answer is not equal to that of the determined Lh value of the blend, the possible precision depends moreover on the relative potencies of the toxoid under test and of the measuring toxin. Table IV illustrates a preliminary orientating experiment to determine the Lh dose of a toxoid by means of a blend of equal parts of the toxoid and of a toxin the Lh of which is 0.058 c.c. In this instance the volumes of blend added to successive tubes

TABLE IV

*Calculation of the combining power of toxoid from the Lh value of a toxin-toxoid blend (McClean's method)*

Observed Lh of blend (c.c.)	0.058	0.10	0.115	0.132	0.152	0.176
Calculated Lh of toxoid (c.c.)	0.058	0.116	0.192	0.27	0.36	0.45

differed by about 15 per cent throughout the series. The precision of the calculated Lh dose of the toxoid is seen, however, to vary at different points in the series. Between the first and second tubes the difference is 32 per cent, whereas between the fourth and fifth tubes it is more than 100 per cent. The potency of the toxoid having thus been roughly ascertained, a closer approximation to the true value may be obtained by retesting at finer intervals. The lack of precision that results if the toxoid is of much lower potency than the toxin may be to some extent obviated in the second test by suitably diluting the toxin till its test dose approximates to that of the toxoid as calculated from the preliminary test.

*Burnet's method and its modifications.* Methods for the determination of the combining power of staphylococcal toxoid have also been published by Burnet (1931), Parish and Tark (1932), Dolman and Hitching (1935) and H. Schmidt (1937). A method essentially similar to these has been used in this laboratory.

Graded amounts of the toxoid under test are added to a series of tubes each containing 1 unit of antitoxin. These are brought to constant volume with saline and the mixtures are allowed to stand on the bench for one hour, after which a small volume of dose of toxin is added. The test cells are added immediately after the toxin and the mixture is incubated after inactivation for 1 hour at 37°C. and for 18 hours at room temperature.

Although in these methods the antigen is added first to the antitoxin, yet since only a small antigen is added to each and only a small proportion of the dose of toxin is added, the results will be slight. Were this the only method, a factor of 100 would be required to obtain the combining power of staphylococcal toxoid as compared with

estimations by the methods of McClean, Dolman and Kitching, Smith and Price to give answers that were the same or nearly the same. That this is not so in practice is shown by table V which gives the results of a study of the combining powers of 5 toxoids and of their toxins of origin by a variety of methods. A search for further causes of discrepancy was therefore made.

TABLE V.

*Estimates of combining power of staphylococcus toxin and toxoid by different methods*

Series no	Antigen	Lh	Lb (Nélls)	Lb (McClean)	Lb (Smith-Price)		Lb (Dolman-Kitching)		Lf	Lf (McClean)
					Indicating dose		Indicating dose			
					10 M H D	2 M H D	10 M H D	2 M H D		
1	Toxin 46	0 09	0 045	—	0 068	0 09	0 044	0 08	0 059	—
2	„ 20	0 095	0 045	—	0 055	0 095	0 032	0 09	0 044	—
3	„ 50 D	0 13	0 08	—	0 088	0 11	0 069	0 08	0 069	—
4	Toxoid 46 b	—	0 085	0 26	0 088	> 0 30	0 083	> 0 30	0 059	0 2
5	„ 20 b	—	0 09	0 30	0 12	> 0 30	0 069	> 0 30	0 062	0 2
6	„ 46 a	—	0 145	0 34	0 18	> 0 40	0 12	> 0 40	0 152	0 4
7	„ 20 a	—	0 20	0 57	0 38	> 1 0	0 27	> 1 0	0 22	0 3
8	„ 50 Da	—	0 53	0 80	0 52	> 1 0	0 30	> 1 0	0 27	0 3

*The displacement of toxoid by toxin from its combination with antitoxin*

Burnet investigated the power of staphylococcus toxin to displace toxoid from its combination with antitoxin. He concluded that, though the degree of displacement was very small, yet it was sufficient to affect seriously the estimate of combining power of toxoid unless the addition of cells was made within two minutes of the addition of toxin. Dolman and Kitching also think it essential to lose no time between the addition of toxin and of cells; they go so far as to advocate the addition of cells before the toxin.

It was of interest to study the effect of varying the amount of toxin used as indicating dose. From table II it is clear that the smaller the indicating dose the less will be the Danyasz displacement. On the other hand a very small indicating dose might be completely used up in displacing toxoid from its combination with antitoxin, whereas if the indicating dose were larger some might be left over and still be available as an indicator of hæmolysis. In a series of estimations by the Smith-Price method the tests were repeated three times using three different indicating doses of toxin. The influence of the Danyasz effect can best be studied in those series which involve toxin only (table V, series 1-3), since in these there are no complications due to displacement of toxoid by indicating





the toxin and antitoxin time to combine before adding the cells. In this connection, H. Schmidt (1937) recommends a contact time of more than 10 minutes. Our latest experiments suggest that the time of contact of indicating toxin and antitoxin in toxoid-combining-power determinations should be at least 3 minutes, and it is probably advisable not to leave the mixtures in contact longer than about 10 minutes, as this may involve an increased risk of displacement of toxoid by indicating toxin. Even with a contact time of 30 minutes, however, the error due to this cause is not great.

TABLE VI.

*Speed of combination of staphylococcus toxin and antitoxin*

Time of contact of toxin antitoxin mixtures before adding cells	Hæmolysis produced by varying amounts of toxin (c c) added to one unit of antitoxin.										
	0 038	0 042	0 046	0 050	0 055	0 060	0 066	0 073	0 080	0 088	0 097
30 mins	—	—	—	—	—	—	—	—	p	ac	c
8 "	—	—	—	—	—	—	—	—	tr	ac	c
4 "	—	—	—	—	—	—	—	—	tr-p	ac	c
Cells added immediately after toxin	—	—	—	—	—	—	—	tr	tr-p	ac	c
Cells added immediately before toxin	tr	tr-p	p	p	p	ac	c	c	c	c	c

c = complete hæmolysis

p = partial hæmolysis

ac = almost complete hæmolysis

tr = trace hæmolysis

— = no hæmolysis

### *The flocculation reaction*

Many references occur in the literature to studies of the combination of staphylococcus toxoid and antitoxin by means of the flocculation reaction. This method is recommended (either alone or in conjunction with others) by Dolman and Kitching (1935), Ramon and Richou (1936) and Ramon, Richou and Rouchdi (1937) for the determination of the potency of staphylococcus toxoid. The Lf doses of the eight antigens under investigation are recorded in table V.

It is not easy to assess the value of flocculation tests for the determination of combining power of staphylococcus antigens on account of the frequent occurrence of more than one flocculation zone. This applies to many sera, including the British standard antitoxin. Again, some samples of toxin and toxoid are more prone to give multiple flocculation zones than others. It may be that the other zones are due to antigens other than  $\alpha$  toxin, but if this is so we do not endorse Dolman and Kitching's view that it is always easy



factors, the amount of antigen present and its affinity for antitoxin. If we assume that the flocculation figures give a true measure of the total amount of antigen present, the ratio of the Lf to the Lb (McClellan) will give an indication of the relative affinities of samples of formol-toxoid and of their toxins of origin. The figures are given in the last column of table V. It will be seen that the two toxoids in which this ratio is highest are 20a and 46a and these are the two in which there is no significant difference between the Lf and the Lb (Néls). This indicates that in these two toxoids the Néls method gives a measure of the total antigen and that no displacement of toxoid by indicating toxin can have occurred.

The facts so far are therefore in accordance with v. Dungen's hypothesis, but it must be remembered that this explanation of the Danysz phenomenon is by no means universally accepted. If we assume that the Danysz effect is due to the formation of an overneutralised compound  $TA_2$  in the presence of excess antitoxin, those methods in which the antigen is added in a single dose must be held to give a truer estimate of the combination of antigen and antitoxin to form a neutral compound than methods in which the antigen is added fractionally. This hypothesis would therefore give the palm to McClellan's method, with the Smith-Price and Dolman-Kitching methods not far behind.

However, it has been shown that staphylococcus toxin can to a certain extent displace toxoid even after it has united with antitoxin and it is probable that if the toxin and toxoid compete simultaneously for the antitoxin, as they do in McClellan's method, the distribution of antitoxin will favour the toxin even more. For this reason, the binding doses obtained by this method are probably rather too high.

It has been suggested by Burnet and by Dolman and Kitching that the displacement of toxoid by toxin is rapidly progressive and that it is therefore essential to leave toxin in contact with the toxoid-antitoxin mixtures for the shortest possible time. We have shown that the evidence brought forward by these workers can be explained on other grounds and we suggest that Dolman and Kitching's results may be too low because they have not allowed sufficient time for the indicating toxin to unite with any free antitoxin before the addition of the cells. On the other hand, it is true that a certain amount of displacement does occur, as indicated by the anomalous results obtained by the Smith-Price and Dolman-Kitching methods when the indicating dose is less than 5 M.H.D. For this reason we do not think that the correction applied by Dolman and Kitching for the amount of antitoxin used up by the indicating toxin can have quantitative significance unless one ascertains the proportion of the indicating dose that has been used in displacing toxoid and the proportion that is free



medium used for 46b was less suitable for immunising purposes. It may well be that the quality of the antigen is at least as important for antitoxin production as the actual amount of antigenic material present.

### SUMMARY.

1 The Danysz phenomenon has been demonstrated in staphylococcus toxin-antitoxin combination

2 The effect is greatest when the two additions of toxin are equal in magnitude

3 The Danysz effect was not encountered in the flocculation reaction.

4 The influence of the Danysz effect on estimations of combining power is discussed

5. Methods in use for the estimation of combining power are critically examined

It is a pleasure to acknowledge our indebtedness to Mr A. T. Glenny and Dr D. McClean for their constructive criticism and help

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576 809 8 576 851 5 (C diphtheriae)

## OXYGEN UPTAKE OF WASHED SUSPENSIONS OF *C DIPHTHERIAE* IN THE PRESENCE OF GLUCOSE AND GLYCOGEN

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SINCE the early days of bacteriology it has been customary to observe the ability of micro-organisms to produce acid from carbohydrates added to the medium in which they are growing. These "sugar reactions" have been used in the identification and classification of varieties and have been of great value in practical diagnosis and problems of bacterial taxonomy. They have, however, been of little value in elucidating the nature of the bacterial variations associated with changes in virulence and invasiveness of different types, and they throw no light on the mechanisms of pathogenicity. This is partly due to the fact that many of the sugars employed could play but little part in the normal ecology of the organisms, ability or failure to ferment them could have no direct bearing on the normal metabolism of the pathogenic bacteria as they play no part in the physiology of the mammalian host on which the bacteria are dependent.

In the *Corynebacteria* group there does appear to be some correlation between pathogenicity and power to ferment carbohydrates. *Gravis* strains of *Corynebacterium diphtheriae* can be distinguished almost invariably from *mitis* by their power of fermenting glycogen and starch. There can be no doubt as to the importance of glycogen in mammalian physiology, while knowledge of the importance of polysaccharide substances in relation to bacterial virulence is being rapidly extended. In these experiments we hoped to see whether it would be possible to correlate the extra virulence of the *gravis* strains with the possession of an extra enzyme system for carbohydrate breakdown. To study the systems more readily and at greater concentrations, washed suspensions of 24-hour growths of *C. diphtheriae* have been used, and this paper gives an account of the oxygen uptake of such suspensions of *gravis* and *mitis* types in the presence of glucose and glycogen.

### METHODS

Only strains which could be readily classified as *gravis* or *mitis* were used (Anderson *et al.*, 1931; Cooper *et al.*, 1935 '36). The *gravis* strains

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gave typical rough, irregular "daisy-head" colonies on solid media, granular growth in broth with marked pellicle formation and always fermented glycogen in fermentation tubes containing the media described below. The *mitis* strains gave smooth round colonies on solid media, produced a uniform turbidity in broth with little or no pellicle and never fermented glycogen in fermentation tubes. Stained films of the *gravis* strains usually showed short forms with few metachromatic granules, while the *mitis* strains usually showed long forms with well-marked metachromatic granules.

Two media were used for growing the organisms—peptone broth prepared as described by Wright (1933) and the semisynthetic medium described by Pope (1932) the composition of which is as follows.

Magnesium sulphate . . . . .	0.2 g.
Calcium chloride . . . . .	0.1 g.
Di-sodium hydrogen phosphate . . . . .	1.0 g.
Di-potassium hydrogen phosphate . . . . .	1.0 g.
Glacial acetic acid . . . . .	3.0 g.
Difco proteose peptone . . . . .	20.0 g.
Water . . . . .	to 1.0 l.

Pope's medium was adjusted to pH 8.0 and filtered and then sterilised by autoclaving, glucose being subsequently added in 0.2 per cent concentration. For study of the fermentation reactions 0.5 per cent. of glucose, glycogen or starch was added instead.

The media were distributed in Roux bottles, 250 ml per bottle. About 1 ml. of a 24-hour growth in broth was used as an inoculum. After incubation for 24 hours at 37°C the culture was decanted into centrifuge tubes and the organisms were spun down and washed twice with 0.8 per cent sodium chloride solution. They were then re-suspended in saline and standardised roughly by an opacity tube so that 1 ml of suspension contained  $50 \times 10^9$  organisms, which corresponded to 4.7 mg dry weight of bacteria. Three Roux bottles usually gave 20 ml of such a suspension. The oxygen uptake was measured with Barcroft manometers as described by Dixon (1934). Each vessel contained 1 ml. of suspension and 2 ml of Ringer phosphate to which appropriate amounts of substrate were added. The composition of the Ringer phosphate was as follows.

Sodium chloride . . . . .	7.08 g.
Potassium chloride . . . . .	0.20 g.
Sodium bicarbonate . . . . .	0.12 g.
M/10 phosphate buffer adjusted to pH 7.6 . . . . .	1.00 l.

The glucose and glycogen were added in final concentrations of 0.2 and 0.5 per cent respectively, as preliminary experiments showed that the minimum substrate concentrations required to saturate the respective enzyme systems lay well below these figures. The glycogen used was a highly purified preparation kindly given us by Dr D. T. Bell, and the same specimen was used for making up the media for the fermentation tubes. All estimations were done in duplicate and the duplicates agreed within 7 per cent. The vessels were shaken in a water bath at 38°C. After a preliminary period of 30 minutes for equilibration, readings were taken and the estimations of oxygen uptake were made over the next 60 minute period. To facilitate comparison of the results of different experiments the oxygen uptake was expressed as the number of cmm of oxygen taken up per hour per mg dry weight of bacterial suspension, referred to as  $qO_2$ .

## RESULTS

The uptake of oxygen in the presence of glucose and glycogen was first determined for two typical corynebacteria, D 13, a *gravis* strain with typical colonies which constantly fermented glycogen, and D 14, a *mitis* strain which never fermented glycogen. The washed suspensions were prepared from 24-hour peptone broth cultures. Table I shows the results from four experiments with each. The  $qO_2$  glycogen for D 13 (*gravis*) varied between 18 and 30, and for D 14 (*mitis*) between 11 and 21. Thus the *gravis* strain had a slightly higher  $qO_2$  glycogen than the *mitis*, but there was not the same hard and fast distinction between the two as in the fermentation tubes.

TABLE I

Oxygen uptake by *gravis* and *mitis* types of C diphtheriae with glucose and glycogen

Expt. no.	Organism.	Control (No carbohydrate)	Glycogen	Glucose	$\frac{qO_2 \text{ glycogen}}{qO_2 \text{ glucose}} \times 100$
26	D 13 ( <i>gravis</i> )	8	18	66	28
31	"	7.5	30	82	37
33	"	11	27	77	35
34	"	13	20	79	33
28	D 14 ( <i>mitis</i> )	4.5	11	88	13
30	"	7	21.5	63	23
37	"	9	16	110	14
38	"	7	13	74	17

In columns 3-5 the figures show the uptake of  $O_2$  in c mm per hour per mg dry weight of suspension ( $qO_2$ ).

Similar determinations were made on a number of *gravis* and *mitis* strains recently isolated by Dr Robinson in Liverpool. In this series the organisms were grown on Pope's semisynthetic medium. It will be seen from table II that, except for M 1, the oxygen uptake in the presence of glycogen exceeded that in the controls with all the *mitis* strains. The ratio  $qO_2$  glycogen  $qO_2$  glucose is of the same order in the two series.

There is thus a discrepancy between the behaviour of the washed suspensions and that of the organisms growing in fermentation tubes. These experiments demonstrate that both *gravis* and *mitis* strains after 24 hours' growth possess a mechanism for glycogen metabolism, but that in the conditions in the fermentation tubes this is apparently not used.

Two possible explanations of this discrepancy suggested themselves: (a) that the *mitis* strains were really capable of breaking down glycogen in the fermentation tubes, but without production of acid, (b) that the clear cut difference between the two types



with regard to acid production might depend upon special conditions of aeration present in fermentation tubes

TABLE II.

*Oxygen uptake of C diphtheriae with glucose and glycogen.*

Organism	Control (No carbo- hydrate)	Glycogen	Glucose	$\frac{qO_2 \text{ glycogen}}{qO_2 \text{ glucose}} \times 100$
<i>Gravis</i> 1	4	34	104	33
" 2	2	18	70	36
" 3	18	47	72	65
" 4	3	21	74	28
" 5	5	24	122	20
<i>Mitis</i> 1	4	6	72	8
" 1	6	7 5	92	8
" 2	12	48 5	132	36
" 2	4	15 5	83	19
" 4	14	29 5	130	23
" 4	4 5	32	123	26
" 6	3	17	74	22
" 6	10	29	120	24

Figures as in table I

*Breakdown of glycogen in fermentation tubes.*

It seemed possible that the difference in behaviour between washed suspensions and growing organisms was only apparent, since if *mitis* strains broke down glycogen in fermentation tubes,

TABLE III.

*Destruction of glycogen by C diphtheriae in fermentation tubes.*

Organism	Total carbohydrate remaining (mg per ml)
<i>Gravis</i> 1	1 6
" 3	1 3
" 4	0 8
" 5	0 9
<i>Mitis</i> 1	2 2
" 2	2 3
" 4	2 3
" 6	2 1
None (control)	2 3

Medium = Pope's semisynthetic.

Period of incubation = 5 days at 37° C

but at the same time produced alkali then acidity would be of no help as an indicator of glycogen breakdown To test this point, a series of tubes each containing 5 ml of Pope's semisynthetic medium and 2 per cent glycogen was inoculated with different



between the two types. Washed suspensions of both *gravis* and *mitis* strains after 24 hours' growth in liquid media are able to take up oxygen in the presence of glycogen, though the *gravis* strains are certainly more active on the average. But under conditions where growth is taking place in the presence of peptone this mechanism would appear to be completely inhibited with the *mitis* type. It has been shown that this inhibition is real, since *mitis* strains do not remove glycogen in fermentation tubes. It has also been shown that conditions of aeration are important in determining fermentation. It is probable that other factors play a part, and possible that in the presence of a nutrient medium containing the products of protein breakdown the metabolism of polysaccharide may be "spared" to a greater extent with *mitis* than with *gravis* strains.

#### SUMMARY.

The oxygen uptake of washed suspensions of *C. diphtheriae gravis* (6 strains) and of *mitis* (7 strains) has been determined with glucose and glycogen as substrates. The  $qO_2$  for glucose was of the same order in both types—about 92. The average residual  $qO_2$  for both types in the absence of added substrates was about 7.5, with glycogen the average for *gravis* strains was 27, for *mitis* strains 19. It has been shown that the distinct difference between *gravis* and *mitis* strains as judged by their power to ferment glycogen in fermentation tubes does not hold for washed suspensions. The possible significance of these results is discussed.

We wish to thank Professor H. R. Dean for his interest and helpful advice and Dr D. T. Robinson for kindly supplying most of the organisms used.

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# THE METABOLISM OF WASHED SUSPENSIONS OF *C. HOFMANNII*

R. PASSMORE \*

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Since the original description of *C. hofmannii* by von Hofmann-Wellenhof in 1888, its clinical significance and classification have been matters of dispute. The whole question of its relationship to *C. diphtheriae* has been reopened by the recent claims of Dold and Weigmann (1934 '35) to have converted *C. diphtheriae* into *C. hofmannii* by the action of human saliva. If this is substantiated the importance of this organism in the epidemiology of diphtheria may be considerable. The complete absence of the power to ferment carbohydrates has always sharply distinguished *C. hofmannii* from other corynebacteria and it was thought that an investigation of this deficiency by means of experiments with washed suspensions of bacteria would be interesting. An organism that cannot utilize glucose is of itself a curiosity, and it was hoped by comparisons with *C. diphtheriae* to throw light on its true classification, and also on the extent of the changes involved in the conversion reported by Dold and Weigmann.

## Methods

The methods used were essentially those described in the previous paper (Knox and Passmore 1934). The strain of *C. hofmannii* was from the National Collection of Type Cultures. Morphologically it consisted of short, stout, rounded rods remarkably uniform in size and with no intracellular granules. It was Gram positive. There was no fermentation of any carbohydrate tested and the organism was completely aspidiot to glucose in heavy doses.

It was found to grow more profusely on solid than on liquid media and to have a somewhat long lag phase; since comparatively little growth had taken place at 24 hours. The details of the growth conditions for each experiment are as follows:

Ex. no.	Medium	Duration of growth
61	Peptonized beefsteak	48 h. - 72 h.
92, 94, 95, 97	Nutrient agar	24 h. -
100, 103 and 111	"	24 h. -
104 and 105	Peptonized beefsteak	24 h. -

\* Growth in Peptonized Beefsteak

between the two types. Washed suspensions of both *gravis* and *mitis* strains after 24 hours' growth in liquid media are able to take up oxygen in the presence of glycogen, though the *gravis* strains are certainly more active on the average. But under conditions where growth is taking place in the presence of peptone this mechanism would appear to be completely inhibited with the *mitis* type. It has been shown that this inhibition is real, since *mitis* strains do not remove glycogen in fermentation tubes. It has also been shown that conditions of aeration are important in determining fermentation. It is probable that other factors play a part, and possible that in the presence of a nutrient medium containing the products of protein breakdown the metabolism of polysaccharide may be "spared" to a greater extent with *mitis* than with *gravis* strains.

#### SUMMARY.

The oxygen uptake of washed suspensions of *C. diphtheriae gravis* (6 strains) and of *mitis* (7 strains) has been determined with glucose and glycogen as substrates. The  $qO_2$  for glucose was of the same order in both types—about 92. The average residual  $qO_2$  for both types in the absence of added substrates was about 7.5, with glycogen the average for *gravis* strains was 27, for *mitis* strains 19. It has been shown that the distinct difference between *gravis* and *mitis* strains as judged by their power to ferment glycogen in fermentation tubes does not hold for washed suspensions. The possible significance of these results is discussed.

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for glucose as with *mitis* strains of *C. diphtheriae* for glycogen (Knox and Passmore). There is no fermentation of the carbohydrate by the organism growing in fermentation tubes and yet washed suspensions of the bacteria have considerable power to break down the carbohydrate. The fact that the negative result in the fermentation tubes is a true value and that actual breakdown of

TABLE II

Effect of addition of dead *C. diphtheriae* on oxygen uptake of washed suspensions of *C. hofmannii*

Expt no	Suspension used	Control (Norm. hydrog.)	Glucose	Glucose
103	<i>C. hofmannii</i> + washed suspension of M 4 heated at 70° C for 30 mins	11.9 29.6	37.1 73	125
110	<i>C. hofmannii</i> + washed suspension of M 4 heated at 60° C for 30 mins	5.7 8.0	25.5 21.1	86
	<i>C. hofmannii</i> + washed suspension of G 5 heated at 60° C for 30 mins	8.5	26.6	

Figures as in table I

glucose has not been masked by an excessive production of ammonia causing alkalinity is shown in table III. Determinations of the total glucose content of the medium by Pirie's (1936) method show that after seven days' growth no appreciable amount of

TABLE III

Destruction of glucose in fermentation tubes by *C. hofmannii* and *C. diphtheriae*

Material examined	Percentage glucose concentration in culture after seven days incubation at 37° C.
Uninoculated medium	0.53
<i>C. hofmannii</i>	0.51
<i>C. diphtheriae</i>	0.53
	0.33
	0.31

Uninoculated control medium contained 0.55 percent glucose. This confirms the observations of Wee have dextrose, mannose, ornithine and fourteen days' culture. Fungal growth of *C. diphtheriae* could find no utilisation of glucose or maltose after

Table III  
as subject

As far as could be determined there was no difference in the properties of washed suspensions of bacteria grown in these various ways

## RESULTS.

### *Oxidation of carbohydrates by washed suspensions*

Table I gives the oxygen uptake for various carbohydrates. The concentration of substrate in each case was 0.05 molar except for glycogen, with which it was 0.5 per cent. The high values for lactic acid show that there is no deficiency in the general oxidising systems. Despite the absence of any fermentation of carbohydrates, there is a considerable uptake of oxygen with glucose, galactose, maltose and glycogen, although the corresponding values for *C. diphtheriae* (except for glycogen) are much higher. With lactose and sucrose the uptake does not exceed that in the control. These results show that qualitatively the enzyme systems possessed by *C. hofmannii* and *C. diphtheriae* are as far as have been investigated identical. The only distinction is quantitative, in the relatively low values of  $qO_2$  for glucose, galactose and maltose given by *C. hofmannii*.

TABLE I.

*Oxygen uptake of washed suspensions of corynebacteria in the presence of carbohydrates*

Expt no	Organism	Control (No carbo- hydrate)	Lactic acid	Glucose	Maltose	Galactose	Sucrose	Lactose	Glycogen
64	<i>C. hofmannii</i>	14.0	100	36					
92	"	6.2	87	27	27	.	8.0	7.1	
94	"	8.8	100	32	32		9.7	8.5	
100	"	7.8	.	27.5	.	25			29
102	"	9.2	.	28		20	6.6		32
104	"	8.5		29	46			10	
Fujita and Kodama (1934)	<i>C. diphtheriae</i>	6	75	75	72	75	5.8	<10	
109	" G 5	4.5	59	100	57	60	5.3	7.5	
111	" M 2	11		63	45	60	11	9	

Figures =  $O_2$  taken up in c.mm. per hour per mg. dry weight of suspension ( $qO_2$ )

The question now arises whether the low oxidative utilisation of glucose is due to lack of a definite enzyme or to absence of a thermostable co-enzyme. To test this point, suspensions of *C. diphtheriae*, heated to 60° and 70° C. for 30 minutes, have been added to *C. hofmannii* in the Barcroft vessels. The results are shown in table II. The extra oxygen uptake has never been greater than could be accounted for by the oxidation of substrates in the killed suspension and no evidence of a co-enzyme has been obtained.

It will be seen that there is the same discrepancy with *C. hofmannii*

for glucose as with *mitis* strains of *C. diphtheriae* for glycogen (Knox and Passmore). There is no fermentation of the carbohydrate by the organism growing in fermentation tubes and yet washed suspensions of the bacteria have considerable power to break down the carbohydrate. The fact that the negative result in the fermentation tubes is a true value and that actual breakdown of

TABLE II  
Effect of addition of dead *C. diphtheriae* on oxygen uptake of washed suspensions of *C. hofmannii*

Expt no	Suspension used	Control (No carbohydrate)	Glucose	Glutamate
103	<i>C. hofmannii</i> + washed suspension of M 4 heated at 70° C for 30 mins	11.8 28.6	37.3 53	125
110	<i>C. hofmannii</i> + washed suspension of M 4 heated at 60° C for 30 mins	5.7 8.5	25.5 25.5	80
	<i>C. hofmannii</i> + washed suspension of G 5 heated at 60° C for 30 mins	8.5	26.5	

Figures as in table I

glucose has not been masked by an excessive production of ammonia causing alkalinity is shown in table III. Determinations of the total glucose content of the medium by Pirie's (1936) method show that after seven days' growth no appreciable amount of

TABLE III  
Destruction of glucose in fermentation tubes by *C. hofmannii* and *C. diphtheriae*

Material examined	Percentage concentration of glucose after seven days' incubation at 37° C
Uninoculated medium	0.33
<i>C. hofmannii</i>	0.34
	0.53
<i>C. diphtheriae</i>	0.33
	0.31

Uninoculated control medium = 0.3% per cent glucose

sugar has been destroyed by *C. hofmannii*. This confirms the observations of Widum (1936), who could find no utilisation of dextrose, mannose, fructose, galactose, lactose or maltose after fourteen days' growth.

#### Oxidation of amino acids

Table IV shows the uptake of oxygen with various amino acids as substrates. The final concentration in each case was 0.05





In the case of *C. hofmannii* the  $qO_2$  for glucose+asparagine is very little above the  $qO_2$  for asparagine, but with *C. diphtheriae* there is marked addition of the two oxygen uptakes. It has not been possible to confirm the observations of Fujita and Kodama (1934) that asparagine accelerates the respiration of glucose. It will be seen, however, that the presence of amino acid has increased the value for  $qC_6H_{12}O_6$  in three experiments. This can be explained by the fact that the respiration in the presence of glucose alone falls off and after two hours may be 10-15 per cent below the initial rate. With added asparagine the respiration rate is level or may even be increased up to 10 per cent above the initial value presumably due to active growth. Thus the presence of amino acids stabilises and may even cause a slight increase of the enzyme for glucose breakdown. Apart from this, the metabolism of amino acids and the metabolism of glucose appear to be completely independent.

TABLE V

Effect of amino acids on the metabolism of glucose

Expt no	Organism	Control	Formate	Asparagine	Glucose		Glucose + formate		Glucose + asparagine	
		$qO_2$	$qO_2$	$qO_2$	$qO_2$	$qC_6H_{12}O_6$ *	$qO_2$	$qC_6H_{12}O_6$	$qO_2$	$qC_6H_{12}O_6$
100	<i>C. hofmannii</i>	7.8	8.5	8.9	27.5	6.7	90	8.6	93	8.9
102		9.2	8.3	4.3	28.4	6.3	87	7.9	95	7.1
105	<i>C. diphtheriae</i> M4	2.9		6.4	80	7.5			114	5.5
107		0.9		100	115	51.5			167	62.0

$qO_2$  as in table I

\*  $qC_6H_{12}O_6$  = c.mm. of glucose (expressed as a gas) destroyed per mg. dry weight of suspension per hour

A further point arises from table V. The ratio  $qO_2/qC_6H_{12}O_6$  gives a value for the number of molecules of oxygen used corresponding to the removal of one molecule of glucose. For *C. diphtheriae* the values are 2.1 and 2.2 (in agreement with Knox and Pasmore) and for *C. hofmannii* 4.4 and 4.5. With neither organism can the whole of the glucose removed be accounted for by oxidations, but the higher value for *C. hofmannii* may be due to the fact that it can oxidise formate. We have obtained a value of  $qO_2 = 4.2$  for *C. hofmannii* with formate and have shown that *C. diphtheriae* cannot oxidise this substrate. Fujita and Kodama have shown that a large percentage of formic acid appears in the anaerobic breakdown of glucose by *C. diphtheriae*, but no data are available as to its aerobic production.

molar (except for tyrosine, which was used in saturated solution). It will be seen that both qualitatively and quantitatively the values are very similar for *C. hofmannii* and *C. diphtheriae*. There is always a high  $qO_2$  for glutamate and asparagine, and for tyrosine and tryptophane the figures are usually slightly above the control value.

TABLE IV.

Oxygen uptake of washed suspensions of corynebacteria in the presence of amino acids

Expt no	Organism	Control	Tyrosine	Tryptophane	Alanine	Asparagine	Glutamate
92	<i>C. hofmannii</i>	6.2	9.8	8.6	20		74
94	"	8.8	15	12	26		110
100	"	7.8				89	85
102	"	9.2		6.7		95	83
104	"	8.5	15			82	
Fujita and Kodaira (1934)	<i>C. diphtheriae</i>	<10	<10	<10	12	47.6	38.8
identical	" G 5	4.5	5.5	3.5	8	70	58
ama (1934) value	" M 2	11	13	17	16	66	53
109	<i>C. hofmannii</i>						
111							

Figures as in table I

Oxygen uptake results fit in with the observations of workers on the requirements of corynebacteria. Thus, Braun, Hofmeier (1929) found that five non-exacting strains of *C. diphtheriae* could use as sole source of nitrogen, aspartate, glutamate, but not any other amino acids. Mueller with a fastidious strain of *C. diphtheriae*, found that asparagine or glutamate were essential for optimum growth. But (1935), working with *C. hofmannii*, found that several amino acids were essential for optimum growth. But whereas 100 mg. of glutamate per 10 ml of medium were needed for maximum growth, tyrosine, methionine and tryptophane, the minimum requirements were less than 5 mg per 10 ml of medium for cysteine.

Effect of amino acids on the metabolism of glucose.

The possibility that the presence of protein breakdown products in the medium may, under certain circumstances, completely inhibit the carbohydrate metabolism of growing corynebacteria, suggested that amino acids might have a marked effect on the metabolism of glucose by washed bacteria. In a series of experiments glucose and amino acid suspensions were added together to the Barcroft vessel and both the oxygen consumed and the glucose utilised were determined. The sugar remaining after the experiment was estimated by the method of Hagedorn and Jensen (1923), after precipitation of the proteins by the addition of 20 per cent phosphotungstic acid in 5 per cent. sulphuric acid to each Barcroft vessel (table V).



## SUMMARY.

These results show the essential similarity of the metabolism of *C diphtheriae* and that of *C hofmannii*. Of the enzyme systems studied, not one possessed by *C diphtheriae* is not also present in *C. hofmannii*. In *C. hofmannii* there is no lack of general respiratory enzymes but the enzymes for carbohydrate breakdown are present in only 25-40 per cent of the quantities in which they occur in *C. diphtheriae* and in addition their activity appears to be completely suppressed in organisms during growth on nutrient media.

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and a surface covering of liquid paraffin. This method is probably the one most commonly used for the production of powerful toxins. If, however, it is desired to follow the quantitative changes occurring in the composition of the cultures during the growth of the bacteria, as we have endeavoured to do in these experiments, a medium containing pieces of meat is not particularly suitable in view of its lack of homogeneity. For this reason we have, as in previous experiments of this kind, preferred the use of ordinary veal broth containing 1 per cent Riedel peptone and 0.5 per cent sodium chloride. Before autoclaving the reaction was adjusted to pH 8.0, afterwards it was found to range generally between pH 7.3 and 7.9. The sterile medium was kept in the ice box in one-litre flasks. Immediately before use 20-30 g sterile calcium carbonate per litre were added and the requisite amount of glucose from a sterile 50 per cent solution. The broth was then carefully boiled and its surface covered with a thick layer of sterile liquid paraffin.

The coli-fermented broth utilised in a number of experiments was prepared by inoculation of the peptone broth with *B. coli* and incubation for 20 hours at 37° C. After heating for a short time in the boiling water-bath, it was filtered through paper, the reaction was adjusted to pH 8.0, and sterilisation by autoclaving followed.

The cultures of *B. histolyticus* were prepared by mixing 1 c.c. of a 24 hours' culture in meat medium with 1 l. of medium and incubating at 37° C. with continual stirring, as described by us in a previous paper (1933).

Table I and fig. 1 show the result of one experiment with *B. histolyticus* upon a medium containing 1 per cent. Riedel peptone to which no glucose has been added.

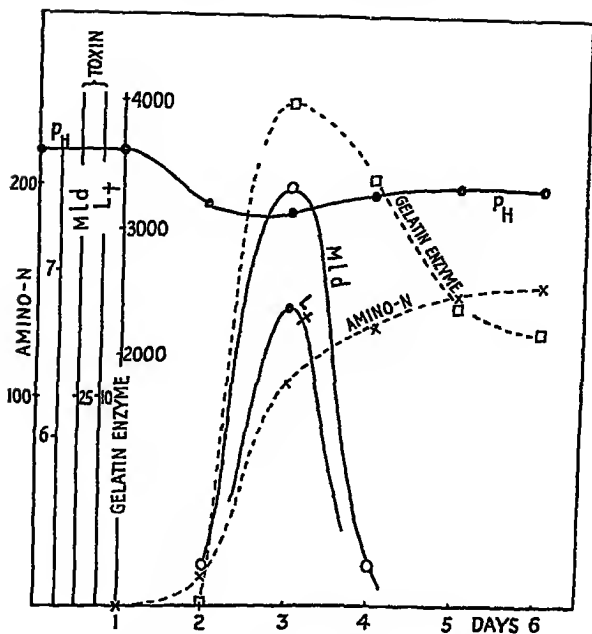
TABLE I.

*Toxin and enzyme production by B. histolyticus (strain 5)  
in peptone broth without glucose.*

Days	pH	Toxin (M. L. D. in 1 cc)	Toxin (L <sub>+</sub> in 1 cc)	Increase of formol- titratable amino-N (mg. per 100 cc)	Gelatin liquefying enzyme (units per cc)
0	7.70	0	0.0	0.0	0
1	7.70	0	0.0	0.0	0
2	7.40	5	0.0	14.0	33
3	7.35	50	14.3	107.3	4000
4	7.45	5	0.0	135.3	3300
5	7.50	0	0.0	149.3	2440
6	7.50	0	0.0	154.0	2220

Medium, 1 per cent Riedel peptone broth

It will be seen from this experiment that under the conditions described no measurable modifications occur in the composition of the medium until the second day, when the pH slowly begins

FIG. 1.—Production of toxin by *B. histolyticus*

to decline and a commencement of enzymatic activity and toxin production (the latter measured by intravenous injections in mice) reveals itself. In the course of the third day the quantity of these substances is greatly augmented, as the toxin and the gelatin-liquefying enzymes reach their highest concentration about 72 hours after inoculation. The concentration diminishes with continued incubation. Meanwhile the albumose digesting action, characterised by the quantity of formal-titratable amino-N, steadily increases.

In this as in all other experiments we have measured both the direct and the indirect toxic value ( $L_{+}$  vis-à-vis 0.005 units of antitoxin), and a glance at fig. 1 will show that the break-up of the toxic and antitoxin-binding substances follows a strictly parallel course. We have carried out similar experiments with other strains of *B. histolyticus* and have found that the course taken by the various processes was identical with that described above, even though the variation in toxin production has naturally been pronounced.

These results are at variance with the findings of other authors



in so far as the time when toxin production sets in is concerned. In our experiments this process commences much later and reaches its maximum after three days, while other workers show that toxin production reaches its maximum after 15-24 hours' incubation. In general the speed of toxin production depends to a very great extent upon the size of the inoculum and on the composition of the medium, as well as on the presence of meat at the bottom of the culture jar. In particular the pieces of meat appear to stimulate considerably both growth and toxin production, presumably owing to the fact that large and continually increasing quantities of split protein substances are placed at the disposal of the bacteria by the action of strongly proteolytic enzymes at an early stage of development of the cultures.

As previously mentioned, attempts have been made to further toxin production by adding glucose to the medium and in this way good results were obtained. We have likewise carried out a number of experiments with varying glucose concentrations as well as with coli-fermented broth. The results of this series are illustrated in tables II-IV.

TABLE II.

*Toxin and enzyme production by B. histolyticus (strain 5) grown in peptone broth containing 0.25 per cent. glucose*

Days	pH	Toxin (M. L. D. in 1 c c)	Toxin ( $L_{+}$ in 1 c c)	Increase of formal- titratable amino-N (mg. per 100 c c)	Gelatin-liquefying enzyme (units per c c)
0	7.65	0.0	0	0.0	0
1	7.65	0.0	0	0.0	0
2	7.40	33.0	10	32.7	400
3	7.45	14.3	0	90.0	2000
4	7.65	2.0	0	130.7	3330
5	7.75	0.0		140.0	2500

Medium as in table I.

TABLE III.

*Toxin and enzyme production by B. histolyticus (strain 5) in peptone broth containing 1 per cent. glucose*

Days	pH	Toxin (M. L. D. in 1 c c)	Toxin ( $L_{+}$ in 1 c c)	Increase of formal- titratable amino-N (mg. per 100 c c)	Gelatin liquefying enzyme (units per c c)
0	7.45	0.0	0.0	0.0	0
1	7.00	2.0	0.0	14.0	28
2	6.45	14.3	3.3	102.2	286
3	6.15	5.0	0.0	116.7	2000
4	6.10	2.0	0.0	121.3	1420
5	6.10	2.0	0.0	130.6	1330
7	6.55	2.0	0.0	144.7	1250

Medium as in table I.

TABLE IV

*Toxin and enzyme production by B histolyticus (strain 5) in peptone broth free from sugar (coli fermented)*

Days	pH	Toxin (M.L.D. in 1 c.c.)	Toxin (L <sub>+</sub> in 1 c.c.)	Increase of formal titratable amino-N (mg per 100 c.c.)	Gelatin liquefying enzyme (units per c.c.)
0	7.90	0.0	0.0	0.0	0
1	7.85	0.0	0.0	9.3	4
2	7.85	14.3	5.0	28.3	28
3	7.60	50.0	14.3	126.0	2630
4	7.05	2.0	0.0	140.0	2630
5	7.75	0.0	0.0	159.0	2220
6	7.90	0.0	0.0	109.0	2000

Medium contained 1 per cent Riedel peptone

In all these experiments growth was satisfactory. Toxin production was greatest and the same in the *coli*-fermented broth (table IV) and in broth without glucose (table I). It was less in the medium containing 0.25 per cent glucose (table II), and still less in the experiment with 1 per cent glucose (table III). Diminishing toxin production with increasing glucose concentration as observed by us is not in accord with the findings of other authors.

In other experiments we have examined the importance of the peptone concentration for toxin production (tables V and VI), no glucose was added to the broth in these instances.

TABLE V

*Toxin and enzyme production by B histolyticus (strain 5) in 2 per cent Riedel peptone broth*

Days	pH	Toxin (M.L.D. in 1 c.c.)	Toxin (L <sub>+</sub> in 1 c.c.)	Increase of formal titratable amino-N (mg per 100 c.c.)	Gelatin liquefying enzyme (units per c.c.)
0	7.70	0	0.0	0.0	0
1	7.05	20	0.7	0.0	0
2	7.35	100	36.7	85.3	2000
3	7.45	20	0.7	100.7	2000
4	7.55	2	0.0	107.3	012
5	7.75	0	0.0	112.0	285

TABLE VI

*Toxin and enzyme production by B histolyticus (strain 5) in 4 per cent Riedel peptone broth*

Days	pH	Toxin (M.L.D. in 1 c.c.)	Toxin (L <sub>+</sub> in 1 c.c.)	Increase of formal titratable amino-N (mg per 100 c.c.)	Gelatin liquefying enzyme (units per c.c.)
0	7.00	0	0.0	0.0	0
1	7.10	200	07.0	109.6	3200
2	7.15	50	14.0	151.0	3000
3	7.35	14	10.0	163.3	2000
4	7.45	14	0.7	171.1	1000
5	7.50	14	3.3	176.0	010

The experiments reveal an increasing toxin production with increasing peptone concentration and they demonstrate at the same time that the larger the quantity of peptone present the earlier is the appearance of toxin production (and possibly also of growth). This is entirely in accord with our observation of early toxin production in media containing pieces of meat.

In Table VII are recorded the results of an experiment of this type on cultures in broth containing pieces of meat but no glucose. From them the fact emerges that toxin production not only sets in very early but that it also assumes much larger proportions than in other experiments where this nitrogen source is lacking.

TABLE VII.

Toxin and gas production by *B. l. typhicus* (strain 5) grown in 1 per cent Riedel peptone broth containing meat.

Time (hr.)	pH	Total viable bacteria (C.F.U.)	Toxin (L.U. in 1 cc)	Increase of formal thymidine nitrogen (mg. per 100 cc)	Gelatin liquefaction (ml. per 100 cc)
0	6.70	0	0	0.0	0
100	6.55	50	25	11.5	100
170	6.17	500	100	48.2	1000
250	5.85	270	100	121.7	2500
375	5.50	330	100	140.0	2500
500	5.20	260	66	168.0	2000
740	5.84	110	50	205.0	1100
1100	6.70	14	5	722.0	285

Finally we have examined toxin production in broth containing Witte peptone and "difco" proteose peptone without the addition of glucose. Growth in both media was satisfactory and so was toxin production with "difco" proteose peptone, while toxin production was rather low in broth with Witte peptone.

#### *The stability of the toxin at different hydrogen-ion concentrations*

A preliminary titration of the toxin of the organisms of group 2 has already examined the importance of the hydrogen-ion concentration for the stability of the toxin of *B. l. typhicus* as well as for the stability of the alkaline digesting enzyme. The results are given already in a full article (Walton and Reymann, 1934). The results obtained with strain 5 are illustrated in Table VIII and IX and in Fig. 2 where the corresponding curves are plotted for the logarithmic scale (Walton and Reymann, 1934). The results are given in Table X as representative data for the stability of the toxin of *B. l. typhicus* at different pH values.

It is seen that the toxin of *B. l. typhicus* is stable at pH 6.0 and 6.5 for 24 hours and at pH 7.0 for 12 hours. At pH 8.0 and 8.5 the toxin is stable for 12 hours and at pH 9.0 and 9.5 for 6 hours.

TABLE VIII

The stability of toxin at different hydrogen ion concentrations  
Strain 5 24 hours at 37° C

Toxin (cc)	N HCl (cc)	N NaOH (cc)	0.9 per cent. NaCl (c.c.)	pH	M.L.D. in 1 cc	L <sub>+</sub> dose in 1 cc
10	0.58		0.42	4.16	5	4
10	0.48		0.52	4.96	100	50
10	0.32		0.68	6.10	200	80
10	0.14		0.86	6.08	107	50
10		0.13	0.87	8.04	143	33
10		0.43	0.57	8.02	100	28

TABLE IX

Effect of reaction on activity of albumose digesting enzyme  
(tested after 11 days at 37° C) Strain 5

Toxin (cc)	20 per cent. Riedel peptone (cc)	N HCl (c.c.)	N NaOH (cc)	0.9 per cent NaCl (c.c.)	pH	Increase of formal titratable amino-N (mg per 100 c.c.)
14	4	1.08		0.02	4.16	4.2
14	4	0.80		1.20	4.06	7.4
14	4	0.40		1.00	6.10	12.6
14	4	0.04		1.96	6.98	14.0
14	4		0.44	1.56	8.04	13.1
14	4		1.00	1.00	8.02	10.7

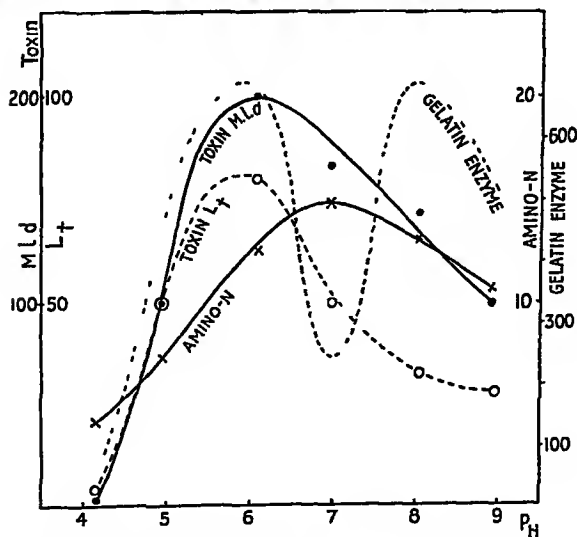


FIG. 2.—Effect of pH on stability of toxin and activity of enzymes of *B. histolyticus*

the albumose-digesting enzyme its maximum activity at about pH 7.0.

### Summary.

1. Under the experimental conditions described, *Bacillus histolyticus* grows luxuriantly in ordinary broth containing 1 per cent peptone irrespective of the presence of glucose

2 Toxin production appears to be greatest in broth without addition of glucose or in coli-fermented broth.

3 The peptone content of the medium has a considerable effect on toxin production, as this increases with increasing peptone concentration. The greatest toxin production occurs in peptone broth containing pieces of meat. This method gives a substantial and steadily increasing production of precisely those protein split products which are of such importance for the growth of the bacteria and for toxin production.

4 *Histolyticus* toxin has its maximal point of stability in the neighbourhood of pH 6.0

5 The optimum reaction for the albumose-digesting enzyme lies around pH 7.0.

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## THE CHEMOTHERAPEUTIC ACTION OF SYNTHALIN IN EXPERIMENTAL INFECTIONS WITH *T BRUCEI* AND *T CONGOLENSIS* \*

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THE therapeutic property of the guanidine derivative, synthalin, in trypanosome infections was described by N and H von Jancsó (1935) for *T brucei* in mice and independently by Schorn and Artinger-Liendo (1936) for *T equinum* in rats. The former were led to investigate this substance by certain theoretical considerations. From enquiry into the mode of action of germanin (Bayer 205), in which there is a long interval before the lethal effect on the trypanosomes follows the injection of the drug, they concluded that this trypanocidal agent acts on the parasites by affecting their sugar metabolism (N and H von Jancsó, 1934 a and b, 1934 35). Schorn (1925) had shown that the vitality of trypanosomes, as manifested by their motility, depended very greatly on the glucose content of the medium in which they were suspended. Also, it had been found by von Issekutz (1933) that when infected animals were treated with germanin and their blood withdrawn a few hours later, the contained trypanosomes showed diminished glycolysis *in vitro*. The von Jancsós (1934 35) observed that trypanosomes which had been subjected to deprivation of glucose *in vitro*, like those which had been acted on by germanin, on introduction subsequently into the body were rapidly phagocytosed by cells of the reticulo endothelial system in the liver, spleen and bone marrow, whereas such phagocytosis did not occur with untreated virulent trypanosomes. They ascertained further that in infected animals treated with germanin certain degenerative changes occurred in the morphology of the parasites, characterised especially by signs of interference with the processes of division. These appearances were seen readily only with animals in which the phagocytic mechanism of the reticulo-endothelial system had been interfered with recently by the combined procedures of splenectomy and intravenous injection of a preparation of colloidal copper—so called reticulo-endothelial blockade. In intact infected animals treated with the drug such degenerative changes were not seen because the trypanosomes at an early stage of degeneration underwent phagocytosis. Accordingly they tested the chemotherapeutic action of certain guanidine derivatives which are known to produce hypoglycaemia. Among these synthalin (decamethylene diguanidine hydrochloride) and synthalin B (the corresponding dodecamethylene compound) are the most effective and have been suggested as substitutes for insulin, although their action is stated to depend on a different mechanism, being associated with toxic effects on the liver (Bodo and Marks, 1928, and others).

\* The expenses of this work were defrayed by a grant from the Rankine Fund

After treatment with synthalin the same phenomena of phagocytosis and degeneration were observed as after germanin. Therefore it was concluded that these guanidine derivatives also act by affecting the sugar metabolism of the parasites. In discussing the means by which this might be brought about, the von Janoscs inclined to the view that synthalin probably does not act directly on the trypanosomes, but leads to trypanocidal effects indirectly through producing a chronic hypoglycaemia in the host and thus causing a "sugar blockade" of the metabolism of the trypanosomes. In the event of this being the mechanism of action, they considered that, in contrast to what holds with other trypanocidal agents, resistance to the action of synthalin might not follow repeated administration. It is specially noteworthy that these guanidine compounds are the only purely organic trypanocidal drugs which do not possess a complex ring structure. In view of the great theoretical importance of the observations and conclusions, further examination of the subject seemed desirable. Since the present work was concluded, observations of Lourie and Yorke (1937) have been published, confirming the chemotherapeutic action of synthalin in trypanosome-infected animals and also showing that *in vitro* it exhibits a trypanocidal activity comparable with that of the aromatic trivalent arsenicals both on normal strains and on strains which had been rendered resistant to atoxyl or germanin.

#### Methods

It is unnecessary to describe the procedures in detail since they are those which have been employed throughout in this laboratory (Browning and Gulbransen, 1934). The trypanosomes used were *T. brucei* (strain Paris III) and a strain, derived by the writer from the latter, which had been rendered fast to arsacetin by treatment with that drug but was not a relapse strain. Also two strains of *T. congolense* were used—one being that previously mentioned (Browning, Cappell and Gulbransen, 1934), the second a strain which when once it appears in the blood of inoculated animals always persists although perhaps with some variations in numbers, till the death of the animal one to several weeks later. All the strains were propagated continuously in mice.

Treatment was given in the form of subcutaneous injections of aqueous solutions of the substances, the dosage being at the rate of 1 c.c. of the dilution shown for a mouse weighing 20 grammes, the animals ranged in weight from 18 to 24 grammes. The guanidine derivative used was synthalin. It was found that usually a single dose of 1:10,000 was tolerated but this amount could not be repeated safely, often subsequent doses of 1:13,000 or 1:15,000 were tolerated when repeated on 4-9 consecutive days. When death or severe illness from larger doses resulted, convulsions seldom occurred beforehand. In some control experiments guanidine carbonate was used, the maximum tolerated dose being 1:200, which could be repeated, while a single dose of 1:100 often produced convulsions and death. The blood sugar was estimated by the Hagedorn-Jensen method, the animals having been quickly anaesthetised with chloroform and the blood taken from the beating left ventricle. Duplicate estimations were always made and close correspondence obtained, 0.1 c.c. of blood being used for each.

#### *Trypanocidal action of synthalin in vivo.*

*T. brucei* infection. Infections with this strain were curable by atoxyl-311 in a single dose of 1:10,000 and in a proportion of animals with 1:20,000, or by arsacetin 1:100, given in each

case 24 hours after inoculation, when scanty parasites were present in the blood, it may thus be regarded as having a fairly normal sensitiveness to drugs. The arsacetin-resistant strain, which under similar conditions as regards treatment was not affected by a dose of 1 40 to 1 50 of arsacetin, was approximately as readily influenced by synthalin as was the non-resistant strain. The von Jancsó, however, obtained the best therapeutic results with a strain of the former type.

The results, which are exemplified in table I, confirmed the curative action of synthalin. With the arsacetin-resistant strain as well as the normal, the sterilising action occurred slowly and it was observed that a preliminary multiplication of parasites took place in the blood whether the first dose of the drug was given five hours before or after inoculation.

TABLE I  
*Effect of synthalin on infection with T. brucei*

Strain	Days						
	1	2	3.	4	5	6	7-120
Normal	S 1 15,000 followed by inoculation 4 hrs later	S 1 15,000 +	S 1 10,000 —	—	—	—	—
Arsacetin fast	S 1 15,000 followed by inoculation 5 hrs later	S 1 15,000 +	S 1 15,000 +	S 1 15,000 —	—	+	—
Arsacetin fast	Inoculation followed about 5 hrs later by S 1 15,000	S 1 15,000 +	S 1 15,000 —	—	—	—	—

In this and the following tables S = synthalin, +, ++, +++ = trypanosomes present in the blood in increasing numbers on the days indicated — = trypanosomes absent as judged by microscopic examination.

No effect on the course of the infection was produced by guanidine carbonate when given alone in a dose of 1 200 five hours before inoculation with the normal strain and repeated on the three following days, or together with a dose of arsacetin 1 200 48 hours after inoculation.

*T. congolense* infection. A distinct therapeutic effect was observed with both strains. This fact is all the more striking in view of the insusceptibility of these trypanosomes toward most chemotherapeutic agents effective against *T. brucei*. Illustrative details are given in table II of a mouse inoculated with strain II, which does not disappear spontaneously from the blood after having once appeared. After the parasites had reappeared on the 13th day the same dose of synthalin (1 15,000) was repeated on the 14th, 15th, 17th, 18th, 20th and 21st days, but without



causing the parasites to disappear from the blood again, also after ceasing treatment they remained abundant. In other animals the parasites after disappearing in response to treatment with the

TABLE II

*Effect of synthalín on infection with T. congolense.*

Days							
1	2-5	6	7	8	9	10-12	13
Inoculated	—	+	+++ S 1 15,000	+++ S 1 15,000	+++ S 1 15,000	—	+

drug reappeared in spite of continued injections throughout the negative phase. An example is given in table III.

TABLE III.

*Effect of synthalín on infection with T. congolense*

Days								
1	4	5	6	7	8	9-14	15	16
Inoculated	+	+++ S 1 13,000	+++ S 1 13,000	++ S 1 13,000	—	— S 1 13,000 each day	+ S 1 13,000	+++

Similar observations were made with the less virulent strain I, a relapse developing for example on the day following the last of nine consecutive daily doses of synthalín 1. 13,000 and after the blood had been free from trypanosomes for seven consecutive days. These results indicate clearly that, contrary to the von Jancsó's expectation, resistance of the infection to synthalín becomes established, although it remains undecided as to whether this occurs owing to alterations in the parasites or the host. Schern and Artagaveytia-Allende, however, state that trypanosomes may become synthalín-fast.

Guanidine carbonate in a dose of 1 200 given on each of three consecutive days had no effect on the established infection with strain II.

*The blood sugar of mice in relation to the  
trypanocidal action of synthalín*

The von Jancsó's have suggested that possibly a slight but persistent hypoglycæmia produced in the animals by synthalín may cause disturbances in the metabolism of the parasites which



TABLE V.

*Effect of guanidine on blood sugar in mice.*

No of doses *	Intervals in hours between doses and examination of blood	Blood sugar per cent
1 (1 200)	4	0 09
3 (1 200 × 3)	51, 28, 4	0 09
4 (1 200 × 4)	70, 56, 28, 4	0 14
3 (1 200 × 2, 1 100)	27½, 4½, 1 †	0 1
4 (1 200 × 3, 1 50)	72½, 49½, 20, 1 †	0 08
4 (1 200 > 3, 1 50)	72½, 49½, 20, 1 †	0 06

\* The amounts of the doses are given in brackets

† Convulsions followed the last dose

clear evidence that the hypoglycaemia produced by synthalin is in itself responsible for its chemotherapeutic action in trypanosomiasis

### Summary

1 The observation that an aliphatic compound, decamethylene diguanidine hydrochloride (synthalin), has chemotherapeutic action in mice infected with *T. brucei* has been confirmed. The therapeutic result was as marked with an arsacetin-resistant strain as with the same strain before being rendered resistant to the drug.

2. Infections due to two different strains of *T. congolense* in mice were also influenced by the administration of synthalin. When repeated doses of the drug were given at daily intervals, however, relapses occurred eventually in the course of the treatment. Relapses were also found to resist further treatment with the drug.

3. Slight hypoglycaemia occurs in untreated mice chronically infected with *T. congolense* but the infection persists in spite of this. The degree of hypoglycaemia is similar to that in uninfected mice treated respectively with synthalin and with guanidine, but only the former of these drugs has chemotherapeutic action. The above facts do not support the view that the chemotherapeutic action of synthalin is due exclusively or chiefly to its effect on the blood sugar of the host. This conclusion is in conformity with Lourie and Yorke's observation that synthalin is highly trypanocidal *in vitro*.

Thanks are due to Mr H E Hornby, Tanganyika Territory, for the second strain of *T. congolense* used in these experiments, and to Messrs Schering-Kahlbaum for placing a supply of synthalin at our disposal.

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## THE ESTIMATION OF THE FRAGILITY OF RED BLOOD CORPUSCLES

E IF CREED

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INVESTIGATIONS were started by the writer some years ago to ascertain whether the adoption of more accurate methods of estimating the resistance of red blood corpuscles to hypotonic solutions of sodium chloride (i.e. of determining the fragility of the red cells) would yield results of interest, and it was hoped that the form of a fragility curve constructed from the data obtained might show distinctive differences in various blood diseases and possibly in other conditions. For the most part these hopes have not been realised, and the only departures from the normal which have been found have been (1) a diminished resistance in acholuric jaundice and some other hæmolytic anæmias, (2) an increased resistance and tendency to lengthening of the curve in other types of anæmia and jaundice and (3) a slight difference in shape of the curve in pernicious anæmia. The main purpose of the investigation has therefore yielded largely negative results, but the method evolved has been communicated to a number of workers and has been employed by them as well as the writer. Some of the results obtained in acholuric jaundice have already been published (East, 1923-24, Dawson, 1931, Vaughan, 1934, 1936). It has therefore been thought desirable to publish the method in detail, with a statement of the principles on which it is based and the findings in normal people. The writer's results in acholuric jaundice and other hæmolytic anæmias will form the subject of a later publication.

### *Method*

Pure sodium chloride is dried to constant weight by heating and allowing to cool in a desiccator. Fifty g. of the dried salt are then accurately weighed out and dissolved in 200 c.c. of distilled water in a measuring flask. The stock of 25 per cent solution of NaCl so obtained will suffice for 200 or more fragility determinations and forms a stable and sterile standard from which the required dilutions are easily prepared. It is essential however, that it be accurately made up and the same care must be bestowed on the subsequent preparation of dilutions from it since very slight errors in the strength of sodium chloride are sufficient to affect the results. The strength of the stock solution may well be checked gravimetrically by precipitation

with excess of silver nitrate. It is of course unsatisfactory to keep weak stock solutions of NaCl owing to the risk of bacterial contamination.

For use, 1 c.c. of the stock 25 per cent solution is diluted to 50 c.c. with distilled water in a measuring flask. The distilled water must be reliable, as the slight acidity of some commercial specimens seriously affects the results. From the resulting 0.5 per cent solution, the required dilutions are made up by the drop method, using Dreyer's dropping pipettes. 24, 25, 26 and so on up to 48 drops of 0.5 per cent NaCl are measured out successively into a series of 25 Wassermann tubes ( $3\frac{1}{2} \times \frac{3}{8}$  ins). The requisite amount of distilled water, 26, 25, 24, 23 . . . 2 drops, is added to each tube to make up the total volume to 50 drops. The contents of each are mixed by inverting the tubes in order on the finger. The tubes now contain 50 drops each of saline of concentration ranging from 0.24 per cent in the first tube to 0.48 per cent in the last, with a difference of 0.01 per cent between successive tubes. This range will be found to cover most fragility curves, except in cases of acholuric jaundice, when higher concentrations may conveniently be prepared from a 1 per cent instead of a 0.5 per cent NaCl solution. In such cases the series should be prepared by taking 42 tubes (or as many as previous experience of the case shows to be necessary), and measuring into them 1, 2, 3 . . . up to 42 drops of 1 per cent saline, each tube is made up to 50 drops with distilled water, giving a series ranging from 0.02 to 0.84 per cent, the tubes differing by 0.02 per cent. Into each of a further 4 Wassermann tubes are measured 50 drops of distilled water. The tubes should be used within 24 hours of being put up, otherwise loss by evaporation will introduce appreciable errors.

Blood is collected from the patient's vein with a dry sterile needle and is allowed to run directly into a wide test-tube  $1\frac{1}{2}$  ins in diameter which has been previously waxed to prevent clotting. Three to five c.c. of blood should be taken, and if more than this has been allowed to flow into the tube the excess should be poured off, as a larger volume will interfere with efficient aeration. A current of air is then blown vigorously over the surface of the blood in the tube by a hand-bellows with a glass delivery tube drawn out to a wide capillary which will reach to the bottom of the test-tube and which is also waxed. The test-tube should be held almost horizontally and should be continually rotated during aeration so as to expose as much blood as possible to the current of air. The aeration should be continued for four minutes.

Two drops of the aerated blood are added with the dropping pipette to each tube of saline and to each of the 4 tubes of distilled water. The contents of each tube are at once mixed by inverting the tube and the mixing is repeated ten minutes later. After a further ten minutes the tubes are centrifuged and the supernatant fluid is pipetted off into a fresh series of tubes (supernatant series).

After washing the corpuscle deposit in the original tubes with normal saline, distilled water is added to make up the original volume. The corpuscle deposit is now lysed by shaking with the distilled water (deposit series).

A series of standard tubes is now prepared. Twenty-one tubes of uniform bore ( $3\frac{1}{2} \times \frac{3}{8}$  ins) are selected with a gauge. The contents of the 4 tubes which contained distilled water only are mixed in a test-tube, and of this mixture one drop is placed in the first of the series of tubes, two in the second, and so on up to the twentieth tube. The contents of each of the 20 tubes are made up to a total volume of 20 drops by the addition of the requisite amount of distilled water and are then mixed by shaking (standard series). Into the twenty-first tube, which has remained empty,

are pipetted the contents of tubes which it is desired to compare with the standards. The colour of the tubes of the supernatant series is matched against the standard series, and the percentage of hæmolytic which has occurred in the varying strengths of NaCl is so obtained, e.g. a tube which matches standard tube 7 has 35 per cent of its corpuscles hæmolytic. In the same way the colour of the tubes in the deposit series is matched against the standard series and the percentage of corpuscles which remain un-hæmolytic in each strength of NaCl estimated.

To estimate with greater accuracy the percentage of hæmolytic where this is less than 20, the first 10 standard tubes are now made up to a total volume of 50 drops by the addition of 30 drops of distilled water\*. A series is so obtained whose colour represents 2, 4, 6, 8 up to 20 per cent hæmolytic. To match tubes of supernatant or deposit series against these weaker standards it is advisable to convert the hæmoglobin into carboxy hæmoglobin by exposure to a current of cool gas and to do the actual matching by looking at a white surface through the depth of the tubes.

The results obtained from supernatant and deposit series should be closely concordant, but the supernatant series will clearly give the more accurate measure where hæmolytic is slight, the deposit series where hæmolytic is nearly complete. The percentage of the total corpuscles hæmolytic of each strength of sodium chloride may be conveniently plotted as a graph.

For most purposes the above method may be simplified as follows:

(a) The original series of NaCl tubes may consist of 12 tubes containing 14, 15, 16 up to 25 drops of 0.5 per cent NaCl solution made up to a total volume of 25 drops with distilled water, each tube will differ from its neighbour by 0.02 instead of 0.01 per cent.

(b) The preparation and matching of the deposit series may be omitted, and the supernatant fluids only matched against the standard series.

(c) The matching of tubes showing less than 10 per cent of hæmolytic and therefore the preparation of the weaker standard series may be omitted.

These simplifications greatly reduce the time required to perform the test and the results obtained usually give all the information required for clinical purposes.

### *Discussion of method*

The following points in connection with the method call for comment.

1 *Use of untreated whole blood.* Some preliminary investigations showed that the susceptibility of red cells to hypotonic saline may be affected by the previous treatment of the blood. Hæmolytic can be produced by purely mechanical means and by hypertonic NaCl solution. If the saline be sufficiently hypertonic, e.g. 14 per cent, it will cause immediate hæmolytic of added blood, but with less concentrated solutions, though there is no immediate hæmolytic, the red cells are damaged in such a way that they become more susceptible to hypotonic saline. If the red cells be suspended in 7 per cent NaCl solution, partial hæmolytic will be caused by subsequently placing them in a 3.5 per cent solution. If blood be added to 7.5 per cent NaCl a trace of hæmolytic will

\* In cases of jaundice where the serum is highly coloured a drop of the undiluted patient's serum should be added to each tube of this weak standard series before matching tubes of the supernatant series against them.



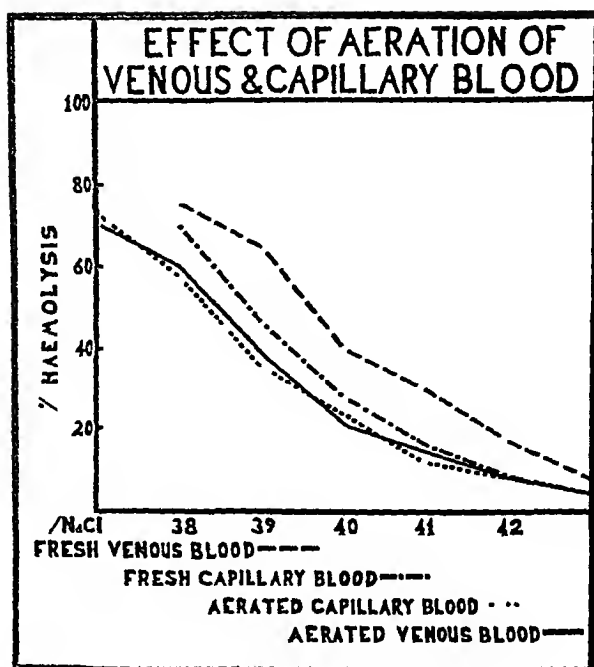


FIG. 1

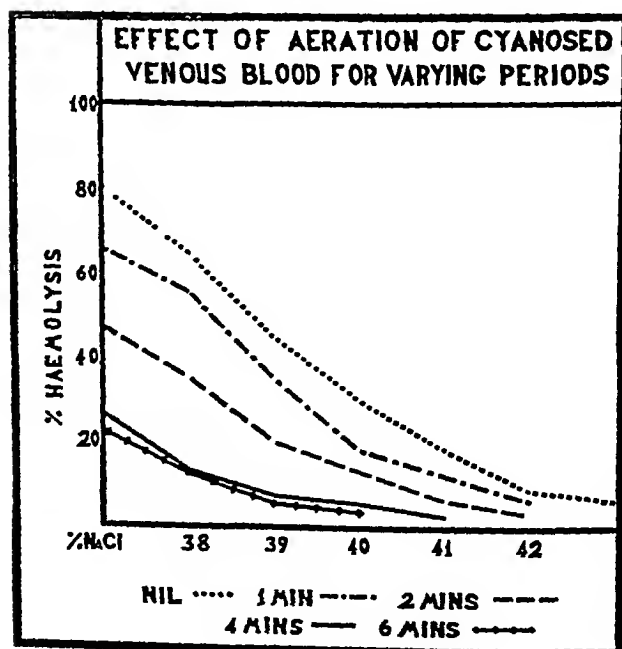


FIG. 2

occur on subsequent exposure to normal saline. To eliminate the risk of altering the red cell fragility it was therefore considered desirable to reduce the preliminary manipulations to a minimum, and untreated whole blood was employed. Some authors have claimed that in certain cases of acholuric jaundice the abnormal fragility can only be detected after the red cells have been washed free from plasma. The writer has therefore made a practice in cases of acholuric jaundice of estimating the fragility both with whole blood and with washed corpuscles. The results have always agreed closely, and three cases of familial acholuric jaundice which showed a normal fragility when whole blood was used still showed no abnormality after the corpuscles had been washed. It is shown below that, even using whole blood, slight acidity of the saline solution makes the corpuscles appear more fragile. If washed corpuscles are used, the very efficient buffering action of the plasma will be lost and slight alterations in pH of the hypotonic saline will produce gross alterations in fragility of the washed cells. This is an additional and strong reason for using whole blood and thereby retaining the buffering effect of the plasma.

2 *Exposure of blood to a current of air*—Investigations were made to determine whether, in the event of venous blood being unobtainable, e.g. in an infant, the substitution of capillary blood would affect the results. Comparative estimations showed that the red cells of venous blood were more fragile than those of capillary blood, but that the difference in fragility could be abolished by exposing the blood to a current of air before adding it to hypotonic saline. It was further found that the longer the tourniquet was applied and the greater the degree of cyanosis induced before the venous blood was collected, the more fragile were the red cells, but again the alteration in fragility produced by cyanosis could be abolished by exposure to a current of air. The essential factor appears to be the reduction of the  $\text{CO}_2$  tension of the blood to that of the atmosphere, and if the blood is aerated before testing it is immaterial whether it is obtained from capillaries or from the vein of a cyanosed limb. Fig 1 shows the effect of the origin of the blood and of preliminary aeration on fragility. To obtain consistent results it is essential that the aeration should be thorough and a strong current of air should be blown over the blood for four minutes. Fig 2 shows the effect of aerating cyanosed venous blood for varying periods.

3 *Amount of blood to be added*—It is important that the ratio of blood to saline should be kept constant, or discrepancies will arise from the varying quantities of plasma introduced. Strictly the effect of the added plasma on the tonicity of the hypotonic saline should be allowed for, but provided the error is kept as constant as possible by adding a constant proportion of blood, it

seems justifiable to ignore it. The increased proportion of plasma in a drop of anæmic blood will make its corpuscles appear less fragile. The proportion of blood to be added—1 drop to every 25 drops of saline—was selected because even with very anæmic blood this gives sufficient colour for matching purposes.

4 *Duration of exposure to hypotonic saline* Hæmolysis by hypotonic saline is very rapid and is practically complete within 5 minutes. The period of 20 minutes laid down above provides an ample margin. No further hæmolysis occurs for some hours, and it is immaterial whether the tubes are centrifuged after 20 minutes or 6 hours, but the centrifugalisation, pipetting off of supernatant fluid and washing of deposit with saline should be completed the same day, as further hæmolysis is liable to occur on standing overnight, even in an ice chest.

5 *Effect of acidity of the sodium chloride solution on fragility.* Some commercial samples of distilled water are acid in reaction and some inconsistent results obtained in the writer's laboratory showing hæmolysis in a normal person up to 0.55 per cent. NaCl were traced to this cause. A series of investigations was therefore carried out to determine the exact effect of acidity on the results (table). It will be noted that there is an appreciable effect with

TABLE.

*Percentage of corpuscles hæmolysed on the addition of one drop of whole blood to 25 drops of hypotonic saline of varying acidity*

HCl in parts per million	Concentration of HCl	Percentage of sodium chloride											
		0.38	0.40	0.42	0.44	0.46	0.48	0.50	0.52	0.54	0.56	0.58	0.60
0.0	0	32	18	5	3	1							
3.6	N/10,000	45	25	8	4	1							
7.2	N/5,000	65	36	25	7	2							
18.0	N/2,000	85	75	45	10	6	4	1					
36.0	N/1,000	95	88	80	40	14	9	7	6	4	2	1	
72.0	N/500	95	95	95	93	80	50	35	25	25	14	7	2

dilutions of HCl extending up to 4 parts per million (N/10,000), even when whole blood is employed. If a 1:100 dilution of blood is used instead of 1:25, the buffering effect of the plasma will be seriously diminished, and with washed corpuscles will be lost. The pH of the saline under these conditions will be a far more important factor.

6 *Effect of anæmia* It has repeatedly been observed in the course of these investigations that anæmic patients tend to have more resistant corpuscles and as the anæmia improves the fragility increases and approaches normal figures. This factor must therefore be taken into account in interpreting the results. A



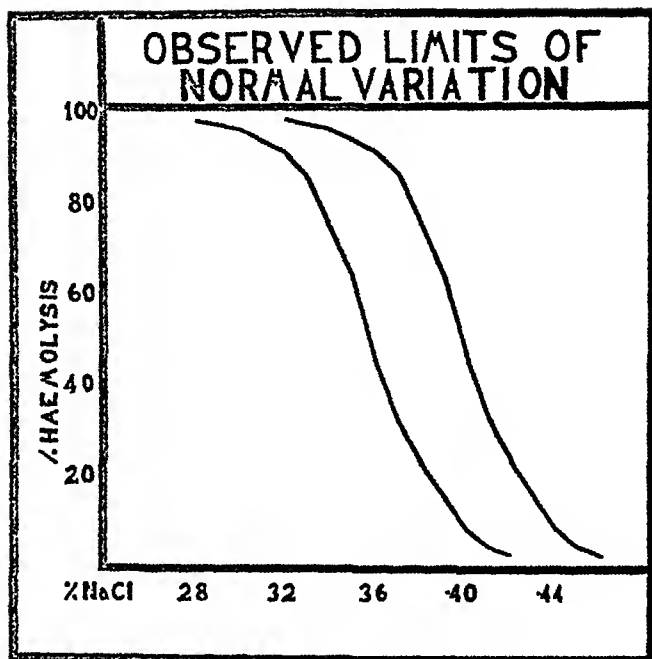


FIG 3.

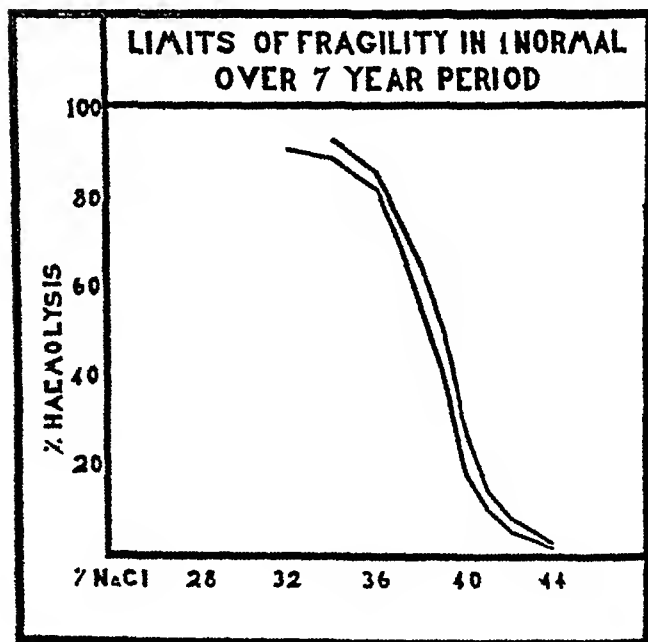


FIG 1.



are added shaken up and then centrifuged. If any hæmolysis is apparent in the supernatant fluid, the case requires further investigation.

### Summary

1. A method of estimating the fragility of red blood corpuscles is described which is believed to give more consistent and accurate results than methods previously employed.

2. The method consists in the addition of measured quantities of aerated whole blood to sodium chloride solutions of varying concentration, prepared by a dropping technique from a stock 25 per cent solution. The percentages of corpuscles hæmolysed and left unhæmolysed are estimated by matching against a series of colour standards.

3. The effect of the venosity of the blood on fragility is considerable, but preliminary aeration of the blood reduces the fragility of samples of varying venosity to a constant level.

4. Variations in the pH of the distilled water employed are a possible source of error in fragility determinations, but their effect is minimised by the use of whole blood in a dilution of 1:25 owing to the buffering action of the plasma.

5. Anæmia *per se* increases resistance to hypotonic saline and this factor must be taken into account in interpreting fragility curves.

6. The commencement of hæmolysis is very gradual and it is impossible to give any point at which it begins or is complete. In normal individuals the concentration of sodium chloride which hæmolyses 4 per cent of the corpuscles varies between 0.41 and 0.45 per cent and the concentration hæmolysing 50 per cent of corpuscles between 0.36 and 0.40 per cent. The fragility curve of a given normal individual remains very constant over long periods.

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175 c.c. capacity previously filled with oxygen. The blood is equilibrated with the contained oxygen by rotating the cylinder rapidly for ten minutes, so that the blood spreads out as a thin film. It is not essential to fill the cylinder with oxygen; air can be substituted and the same results obtained. For this reason it is unnecessary to take elaborate precautions to prevent dilution of oxygen with air when placing the blood in the cylinder prior to aeration. Oxygenated in contradistinction to aerated blood has, however, been used throughout the present study. For routine purposes it is recommended that aeration be employed, using a cylinder with no stopper. With the stopper in place there is produced an apparent slight increase in fragility due to  $\text{CO}_2$  retention.

One drop of oxygenated or aerated blood is then added to each tube of saline and two drops to the tubes containing distilled water. These are mixed by immediate inversion, corked and replaced in the ice chest as soon as possible.

One c.c. of saline corresponds to 22 drops of blood, and therefore the proportion of blood to saline is in the ratio of 1:23. At the end of half-an-hour the tubes are removed from the ice chest and their contents centrifuged at 1-2000 *r.p.m.* for five minutes.

Standards for quantitative estimation of the degree of haemolysis are then prepared by diluting the blood and distilled water solution so that dilutions from 10-90 per cent are obtained at 10 per cent intervals.

The percentage of haemolysis in the different tubes is estimated by direct matching of the haemoglobin-tinged supernatant fluid in the tubes in which haemolysis has taken place with the prepared standards. If the colour of the haemoglobin solution lies between two standards, *e.g.* 20 and 30 per cent., the percentage haemolysis is taken to be midway between the two, *i.e.* 25 per cent. When the haemolysis is obviously only just in excess of that in a standard tube, *e.g.* 20 per cent, this is called 20 per cent +, similarly if slightly less, 20 per cent -. When haemolysis is almost complete it becomes difficult to match the tubes in the way described and readings above 85 per cent should not be considered absolute. If for experimental purposes venous blood or blood saturated with  $\text{CO}_2$  is used, it is necessary to centrifuge the standards, as a precipitate of globulin develops when slightly acid plasma is diluted with distilled water. The amount of precipitate depends on the pH of the plasma.

The curve is recorded graphically by plotting as ordinates percentage haemolysis and as abscissae concentrations of NaCl solution, as has been used by Dawson (1931), Whitby and Hynes (1935), Vaughan (1936, 1937) and Creed (1938). The method of expressing this curve mathematically in any instance is that previously suggested by Vaughan (1937). The median corpuscular fragility (M.C.F.) is that concentration of saline which will cause 50 per cent haemolysis. The shape of the curve or span of resistance is expressed as *b*, a figure representing the slope of the line on which lie the 16-84 percentage haemolysis values expressed as normal equivalent deviations. This latter figure is without great practical significance.

Red cell counts, haemoglobin percentage and mean corpuscular volume (M.C.V.), diameter (M.C.D.) and thickness (M.C.T.) have been estimated by methods previously described (Price-Jones, Vaughan and Goddard, 1935). The difficulties inherent in measuring cell diameters on dry films (Ponder and Millar, 1925) are appreciated, but since the error is probably a constant one, results obtained have a relative value.

The method of estimating red cell fragility above described has proved satisfactory in practice and the results obtained where estimations have been duplicated or triplicated have been notably constant. In order to

determine when an observed change in fragility may be considered significant, 10 estimations were performed on a sample of normal blood with the following results

$$\begin{aligned} * M (MCF) &= 0.3507 \pm 0.0003 \text{ g per cent NaCl} \\ \sigma &= 0.001 \text{ g per cent NaCl} \\ V &= 0.28 \text{ per cent} \end{aligned}$$

For b the figures were —

$$\begin{aligned} M &= 57.2 \pm 1.053 \\ \sigma &= 3.341 \\ V &= 5.841 \text{ per cent} \end{aligned}$$

*The red cell fragility in normal subjects*

Using the above technique fragility estimations have been performed on a series of 50 normal subjects, 26 men and 24 women

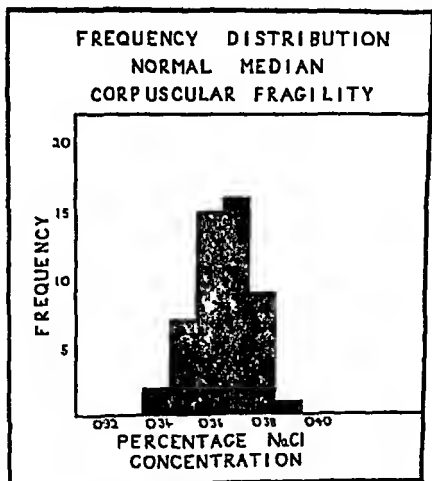


FIG 1

(figs 1 and 2) The calculated figures for the MCF of the whole series were —

$$\begin{aligned} * M &= 0.366 \pm 0.0015 \text{ g per cent NaCl} \\ \sigma &= 0.0105 \text{ g per cent NaCl} \\ V &= 2.9 \text{ per cent} \end{aligned}$$

$$\frac{\sigma}{\sqrt{N}} = 0.0015 \text{ g per cent NaCl}$$

Possible limits  $M \pm 3\sigma = 0.334 - 0.398 \text{ g per cent NaCl}$

---

\* M = arithmetic mean  
 $\sigma$  = standard deviation  
 V = coefficient of variation

The observed limits of M.C.F. were 0.347–0.387 g per cent. NaCl, and the values for  $b$  24.62. The calculated figures were —

$$M = 42.7 \pm 1.2$$

$$\sigma = 8.6$$

$$V = 20.14$$

$$\frac{\sigma}{\sqrt{N}} = 1.2$$

Possible limits  $M \pm 3\sigma = 16.9$ –68.5

Further study of the value of  $b$  as an expression of the shape of the curve in pathological bloods makes it appear probable that it is not in practice very useful. Variations in the slope of the curve except in rare instances tend to affect particularly its upper and lower ends, which are not included in the slope measured by  $b$ .

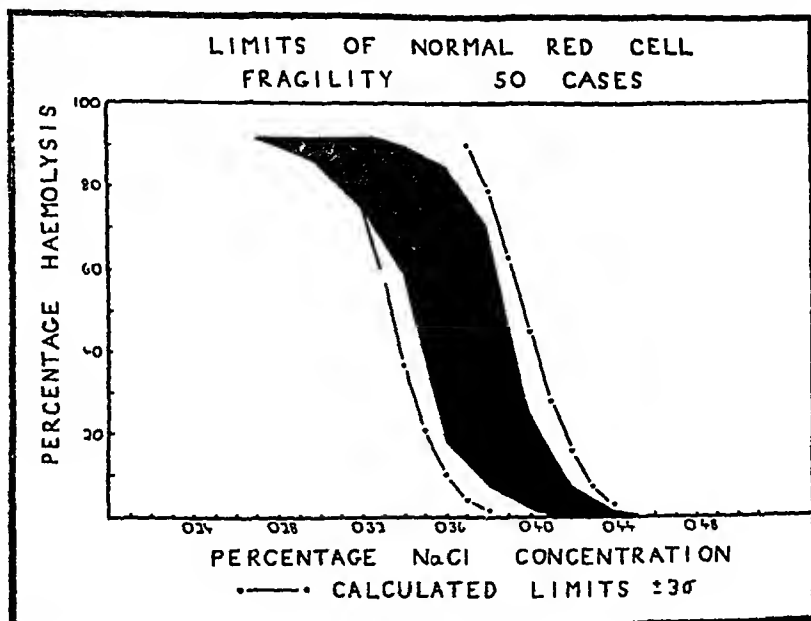


FIG. 2

The calculated mean M.C.F. for the 26 men was 0.366, for the 24 women 0.365 g per cent. Therefore the present small series suggests that there is no significant difference in fragility between men and women, as Leake and Pratt (1925) had tentatively suggested.

Hæmolysis began in concentrations of saline varying from 0.40 to 0.44 g per cent. It will be noted that no figures have been given for complete hæmolysis. This is difficult to determine by the above technique. There are normally present a few extremely resistant cells (Creed) which can be determined if the deposit after centrifugalisation is examined under the microscope. This was done in 30 of the normal subjects. The saline

concentration producing complete hæmolytic was found to vary from 0.10 to 0.22 per cent NaCl

## FACTORS AFFECTING THE RESISTANCE OF THE RED CELLS TO HÆMOLYSIS IN HYPOTONIC SALINE

### 1 Intrinsic factors

#### (a) Red cell dimensions

It has been suggested that red cell resistance to hæmolytic in hypotonic solutions is closely associated with, if not dependent upon, red cell thickness (Vaughan, 1937). A high degree of correlation was indeed found in a miscellaneous group of cases between mean corpuscular thickness and median corpuscular fragility. On the other hand, in certain patients with acholuric jaundice after splenectomy, median corpuscular fragility was still increased while cell thickness returned to normal (Vaughan, 1937). It appears therefore that though cell shape may be an important factor influencing hæmolytic in hypotonic saline solutions it is not necessarily the determining factor. When the probable mechanism of hypotonic hæmolytic is considered the important part which might be played, on theoretical grounds, by cell shape is apparent.

The available evidence, summarised by Ponder (1934), suggests that when red cells are placed in a hypotonic solution, water enters the cells which thereby increase in volume. Ponder and Saslow (1931) believe that at first the red cells may increase in volume without actual stretching of the cell membrane or alteration in diameter, due to partial eversion of the cell's concavities. If the medium in which the cells are suspended is sufficiently hypotonic a further increase in water content is associated with both stretching of the cell membrane and a decrease in cell diameter (Ponder, 1922-23, 1933, Ponder and Millar, 1925). If the tonicity of the solution is sufficiently low the critical hæmolytic volume (Jacobs, 1930) may be reached and hæmolytic will then take place as the membrane is unable to resist further stretching. It follows that before actual hæmolytic takes place the cell responds to an increased water content by adjustment to a more spherical form, thus postponing as long as possible actual stretching of its membrane. The initial degree of spherocytosis present is therefore of importance in determining at what point hæmolytic will occur.

The ratio  $\frac{MCT}{MCD}$  of the red cell gives a measure of how far a cell diverges from the spherical form, and it has therefore been used in the present study as the expression of cell size most likely to be linked with red cell fragility.

Fig. 3 represents graphically the relation between this ratio and the corresponding median corpuscular fragility (MCF) in

a series of 19 cases, both normal and pathological \* The coefficient of correlation is  $+0.86$ , with a probable error of  $0.058$  There is thus a significant correlation between the two variables Here as elsewhere in the present study MCF has been corrected in part for the degree of anæmia present (see p 353)

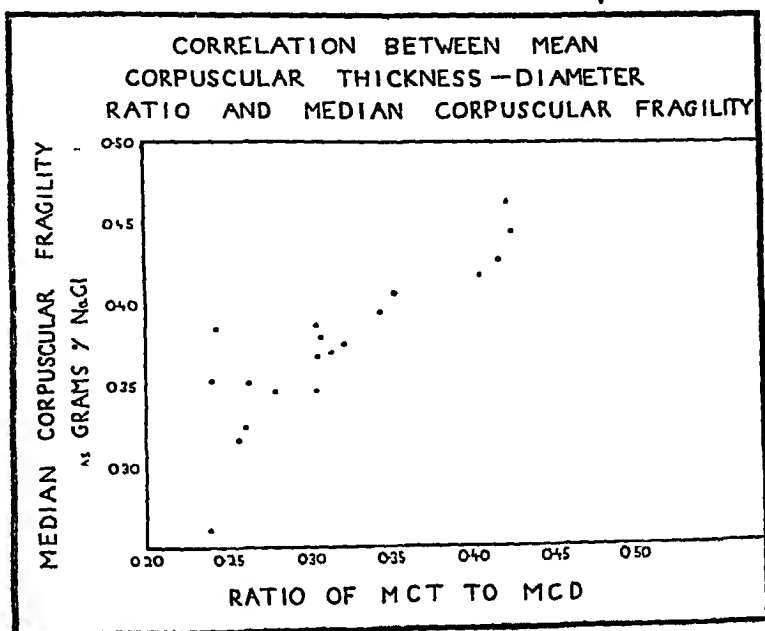


FIG 3

In the same series MCT has been compared to corrected MCF In this case the coefficient of correlation is  $+0.72$ , with a probable error of  $0.11$  The  $\frac{\text{MCT}}{\text{MCD}}$  ratio is thus preferable to MCT alone as the expression of red cell size which may influence red cell fragility.

(b) *Chemical composition of the cell and its membrane.*

As already suggested red cell shape cannot alone be responsible for red cell fragility since alterations in cell shape may occur without affecting the fragility (Vaughan, 1937) Variations in the chemistry of the cell are probably also concerned (Maizels, 1936; Erickson *et al*, 1937, Williams *et al*, 1937) Erickson *et al* found no relationship between fragility and cell protein, mean corpuscular volume or hæmoglobin content The latter finding has been confirmed in the present study

The membrane itself is of possible importance Erickson *et al*,

\* Detailed findings in individual cases are given in an unpublished appendix, the typescript of which has been deposited in the General Library, British Museum (Natural History), London, S W 7.

however, studying the lipid content of the red cells, did not find abnormalities in the phospholipoid-cholesterol ratio associated with abnormal fragility. It is probable that the capacity of the red cell membrane to withstand stretching may vary, but this is a point difficult to determine. Ponder (1934-35) has suggested, however, that the volume of the cell may at least partly determine how great an increase in surface area can take place before rupture.

## 2 Extrinsic factors

It has been shown that the resistance to haemolysis in hypotonic saline of any red cell is dependent, at least in part, upon that cell's dimensions. These control the amount of water which may enter the cell before its membrane is submitted to an increased tension sufficient to produce haemolysis. It is also certain that any physico-chemical process which modifies the ionic and hence the water content of the red cell will necessarily alter that cell's resistance to haemolysis. An increased ionic content requires a greater influx of water to restore equilibrium with an external saline solution of given tonicity. This results in diminished resistance to haemolysis. Similarly a reduced ionic content results in increased resistance.

### (a) *Oxygen and carbon dioxide content of the blood*

The fact that alteration in  $O_2$  and  $CO_2$  content of the blood under test modified fragility has been noted by Butler (1912-13), Whitby and Hynes (1935) and Creed (1938). They have shown that the more "venous" the sample of blood, the more "fragile" its cells will become and vice versa. This undoubtedly is associated with the increase in cell volume which occurs when the  $CO_2$  content of blood is increased.

Van Slyke, Wu and McLean (1923) obtained a 0.4 per cent increase in cell volume with a corresponding increase in  $CO_2$  tension from only 41.6 to 49.2 mm Hg. Doisy and Eaton (1921), however, only obtained a 2.6 per cent increase in volume in association with an increase in  $CO_2$  tension from 23 to 760 mm Hg, whilst Joffe and Poulton (1920-21) could detect no significant increase when the  $CO_2$  tension was raised to 90 mm Hg.

In a series of 21 normal and pathological bloods the fragility of similar samples equilibrated in an atmosphere of  $O_2$  and  $CO_2$  has been compared. In every case the  $CO_2$ -saturated sample showed a remarkable increase in fragility compared with the same blood saturated with oxygen. There was also a parallel increase in packed cell volume. This varied from 9.3 to 20.6 per cent of the original volume of packed cells. The close association between increase in fragility and increase in cell volume can be shown graphically (fig. 4). Here the difference in packed cell volume

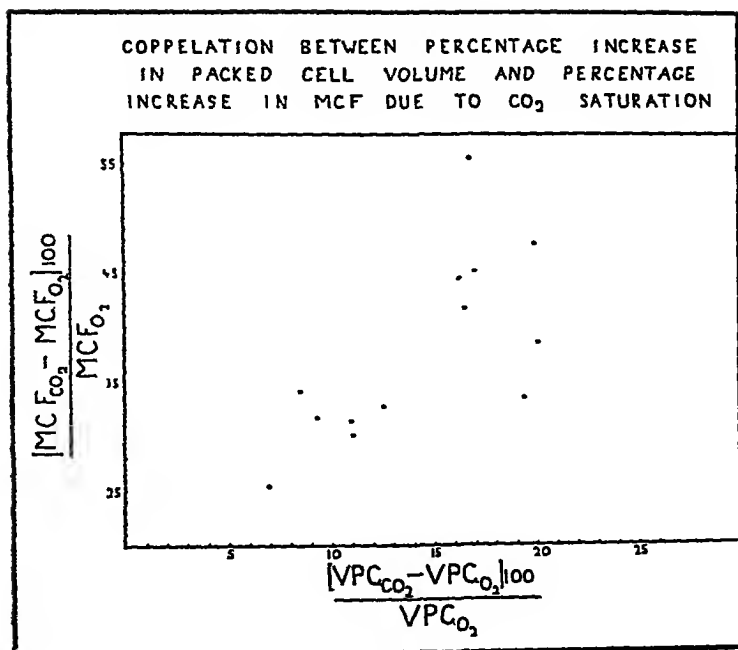


Fig 4

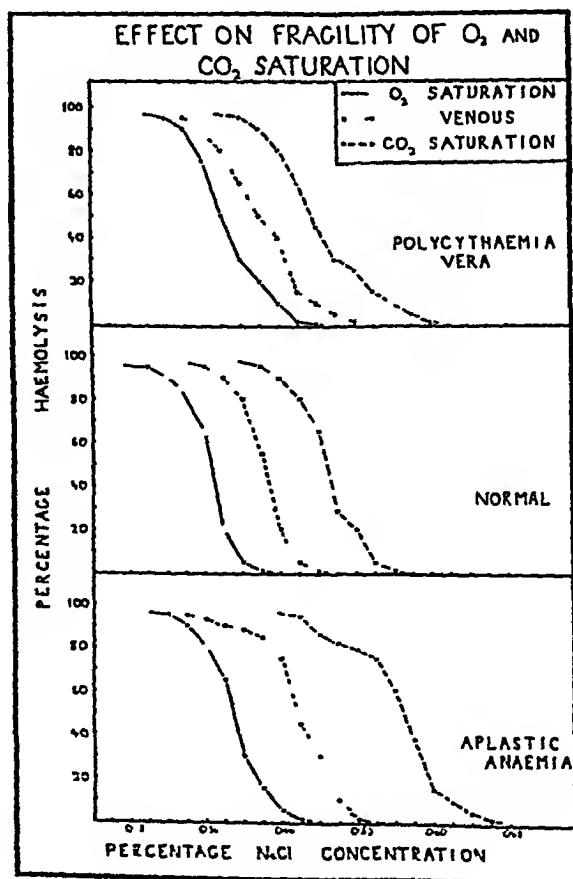


Fig 5





an increased loss of osmotically active substances with associated water from the cells to the hæmolytic solution with increased temperature, resulting in the cell becoming smaller. The cells suspended in a warmer medium are thus able to take in more water before rupturing and hence appear more resistant. The mechanism of this ionic loss is disputed (Ponder, 1934). In the present study these findings have been confirmed. In the systems used it has been found that an alteration of  $2^{\circ}\text{C}$  in the temperature of the saline solution used for hæmolysis was equivalent to about an 0.002 per cent alteration in saline concentration (fig. 7)

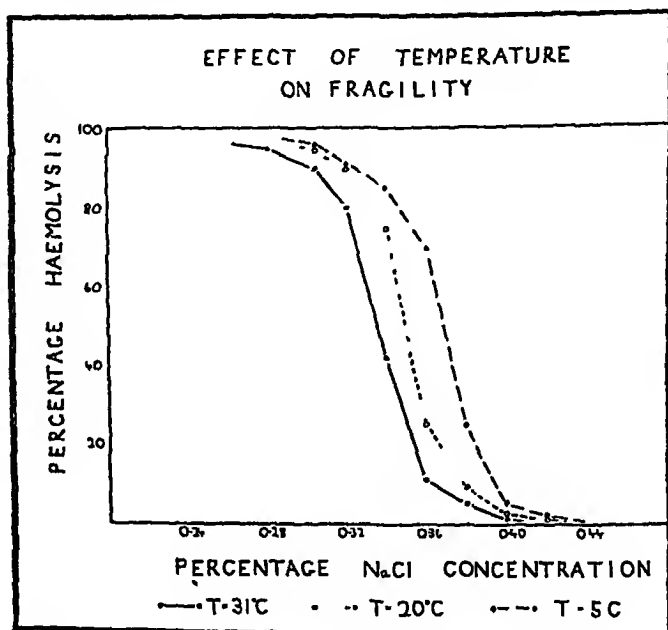


FIG 7

### (c) *pH of saline solution*

Hampson and Maizels (1926-27) have shown that the volume of any red cell suspended in saline depends upon both the concentration of NaCl and the *pH* of the solution. They found that for all tonocities cells have a minimum volume at a *pH* of 8.1, whilst at both higher and lower *pH* the cell volume rises. From this it follows that the degree of hæmolysis at any salt concentration will vary slightly with the final *pH* of the suspension (Jacobs and Parpart, 1931, Saslow, 1932)

In the technique used unbuffered saline has been employed as a hæmolytic solution. This is probably justified, because when whole blood is added to saline in the proportion of about 1:20, the plasma contained in the blood is sufficient to prevent the cells being subjected to any gross alteration in *pH*. In an actual



The observed M C F. at the different cell-plasma dilutions is contrasted with the corresponding red cell count. In order that the effect of anaemia can be justifiably contrasted, irrespective of the level of the original M C F., the logarithms of the mean corpuscular fragilities are recorded. It is at once evident that anaemia has produced an alteration in fragility since in every case the more anæmic the blood the less fragile the cells appear to have become (fig 9)

At least two factors may explain this alteration in fragility

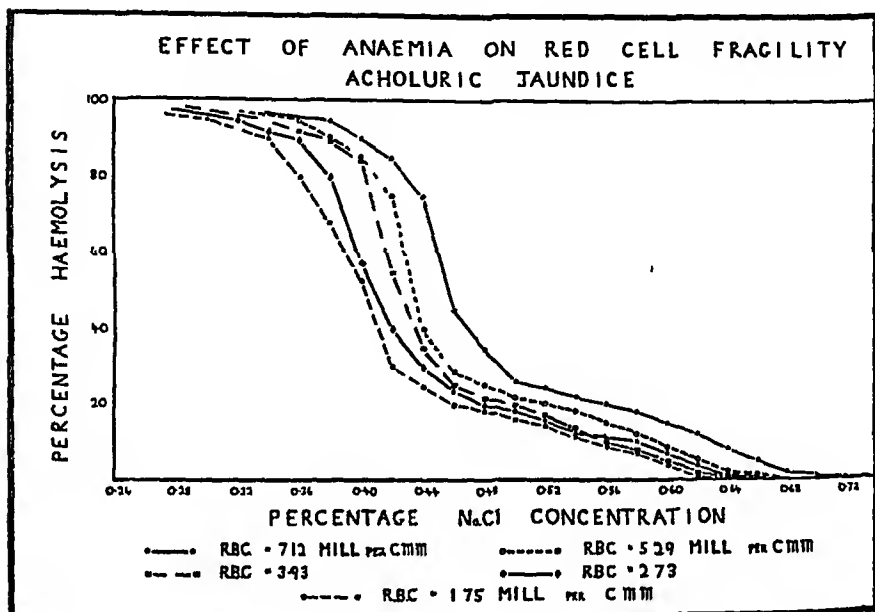


FIG 9

i *Variation in the amount of added plasma* This depends on the fact that when a drop of blood is added to 1 c c of NaCl solution the effective salt concentration is raised because a certain amount of plasma of ionic concentration 150-160 m e q per litre (about 0.90 per cent NaCl; Peters and Van Slyke, 1931) is added. At once it follows that the more plasma there is contained in any drop (as in "anæmic" blood) the greater is the osmotically equivalent NaCl concentration of that drop and the higher is the resulting salt concentration of the plasma-saline hæmolytic mixture.

For example, if one drop of blood of packed cell volume 20 per cent, and a further drop of the same blood with some of its plasma withdrawn so as to have a packed cell volume of 60 per cent, are added to two tubes each containing 1 c c (22 drops) of NaCl solution of 0.38 per cent concentration, a drop of "anæmic" blood is added to one tube and a "polycythæmic" drop to the other. In the case of the former this contains 80 per cent. plasma. Therefore we have added 80 per cent of one drop of 0.90 per cent NaCl to 22 drops of 0.38 per cent NaCl. This will raise the actual salt





published by Lele and Pratt using Sumner's technique. They give wide limits and that hemolysis is occurring as high as about 0.6 per cent. Vaughan and Creed (1927-28) who report complete hemolysis at about 0.24 per cent. Whitty and Hynes also using a modified Sumner's technique have published quantitative curves corresponding to normal limits. They find in a series of 50 men hemolysis commencing at between 0.1 and 0.51 per cent and practically complete at 0.33-0.39 per cent NaCl. The normal limits found by Creed agree quite closely with the present results while Vaughan's (1937) M.C.I. value is slightly greater.

From the preceding observations it is apparent that these varying results probably depend upon differences in technique. These include (1) qualitative differences in the hemolytic solution used, (2) varying temperatures at which the estimations are performed, (3) the fact that in some techniques the red cells are "washed" and that in others whole blood is used, (4) differences in  $O_2$  and  $CO_2$  content of the blood at the time of testing. It is believed that the technique described in the present paper excludes differences due to such possible external factors.

### SUMMARY

1. A quantitative technique for estimating red cell fragility is described.

2. The calculated normal median fragility measured on 50 normal subjects is 0.366 g per cent NaCl the healthy range being 0.334-0.398 g.

3. Red cell fragility is closely related to cell dimensions expressed as the  $\frac{MCT}{MCD}$  ratio. The latter may be termed an intrinsic factor influencing fragility. Increase in this ratio is associated with increase in fragility, but the reverse is not necessarily true.

4. Extrinsic factors influencing fragility are the  $O_2$  and  $CO_2$  tension of the blood and the temperature and pH of the haemolytic solution.

5. Anemia produces an apparent reduction in fragility. This is partly due to the increased amount of plasma contained in any drop of blood, partly to change in cell volume resulting from oxygenation. Chemical variation in the cell constitution may also be important.

We are grateful to Dr F. H. Creed for first demonstrating his method to us, also to Dr A. Gilpin of King's College Hospital, to the chief Medical Officer of the London County Council and to the physicians and surgeons of the British Postgraduate Medical School who have allowed us to investigate their patients. One of us (J. V.) is indebted to the Leverhulme Trust of the Royal College of Physicians for a grant which has enabled this work to be carried out.

haemoglobin present (Henderson, 1928). It follows that the fewer the cells present the more water will each cell lose before equilibrium between cells and plasma is once more established at the new *pH*. Loss of water and anion means reduction in mean corpuscular volume and this in turn results in increased resistance to hypotonic haemolysis. In 6 out of 7 cases investigated this reduction in MCV was demonstrable.

The data recorded in fig. 8 suggest that this effect varies in different pathological conditions. In a normal subject and in two cases of hypochromic microcytic anaemia it was approximately the same, but it was greater in the three other instances (two cases of acholuric jaundice and a case of pernicious anaemia). Hampson and Maizels (1927-28) have shown that in these last two diseases the *pH* of the cells is lower than normal (average 7.201 in 5 cases of pernicious anaemia, average 7.072 in 4 cases of acholuric jaundice, the normal being 7.396 in 10 cases). This increased acidity of the red cells may be associated with an abnormal response when exposed to more alkaline plasma.

It is probable that chemical differences, at present unrecognised, in different types of anaemic red cells may also be responsible for differences in response to haemolytic solutions. The importance of the chemical constitution of the cell is emphasised by the fact that in acholuric jaundice following splenectomy red cell fragility may remain increased while thickness returns to normal. Observed red cell fragility is therefore decreased in anaemia, apart from any fundamental variation in cell size and shape. Changes in the latter are due both to the addition of varying amounts of plasma in any drop of blood and to aeration of the blood under test. Quantitatively this last depends on the number of cells present. It must be realised, however, that the term "corrected MCF" does not imply complete correction for anaemia. Allowance has been made for added plasma only, as the data are too slender to permit further correction.

It is essential, therefore, in performing fragility estimations to consider the degree of anaemia which may be present. The effect of anaemia in reducing red cell fragility is sufficient to influence results obtained by a less sensitive method than the one here used.

#### DISCUSSION.

Previous workers have found variable figures for red cell fragility in normal subjects. Hill (1915) in a series of 19 cases gives limits of 0.475 per cent NaCl for beginning haemolysis and 0.275 per cent for complete, whilst Wiseman and Bierbaum (1931-32), using diluted plasma, give corresponding values of 0.412 and 0.300 per cent (equivalent NaCl concentration) in a series of 50 normal subjects. More recently Daland and Worthley (1934-35) give limits of 0.47 per cent as "trace" of haemolysis and 0.27 per cent as complete in a series of 20 cases. Quantitative limits have been

# VENOUS SPLENOMEGALY—A STUDY IN EXPERIMENTAL PORTAL CONGESTION\*

T. BHASKARA MASON

*From the Pathology Department University of Edinburgh*

(PART XVIII)

THE effect of venous congestion in the production of chronic splenomegaly in man has been variously estimated. It has been argued for instance that the extreme splenomegaly of splenic aneurysm and Banti's disease is the result of primary obstructive lesions of the portal and splenic veins (Dock and Warthin, 1904, Warthin, 1910, Klemperer, 1928, 1936). On the other hand, the fact that the spleen in cardiac stasis never shows such extreme enlargement unless there is a complicating factor such as subacute endocarditis is against this view.

Of pioneer studies on the circulatory disturbances of the spleen, Malpighi's experiment of ligation of the vessels of the spleen in a young dog (quoted by Foster, 1901) was followed by an atrophy of the organ. Ligation of the portal vein has also been carried out, but mostly with a view to determine the effect on the liver. A congestive reaction in the spleen following blockage of the splenic vein was first noted by Basler (1863) and the studies of Soloff (1899) defined the congestive reaction. He found in dogs and rabbits within about ten minutes after obstruction distension of the pulp veins and sinuses. As the congestion increased blood percolated into the meshes of the pulp which he argued was due to the alteration from a "closed" to an "open" circulation. Wickham (1891) found in dogs that venous engorgement produced by constriction of the veins gradually disappeared within 21 days after the operation. The limitations of the congestive enlargement were determined by Warthin who found that after ligation of the splenic veins in dogs and rabbits there was an immediate passive congestion with moderate enlargement of the spleen, this lasted for about six weeks and was followed by irregular atrophy. No evidence of proliferative changes could be obtained. Warthin concluded that the condition of splenic hyperplasia in man due to obstruction by thrombosis of the splenic and portal veins could not be reproduced in the experimental animal. Following ligation of the main branch of the portal vein in rabbits, Rous and Lorrain (1920) did not find any significant splenic changes.

More recently Jüger (1931) has claimed that the effect of partial ligation of the portal vein in dogs is quite different from that induced by ligation of the splenic vein since an intermittent congestion was induced by the

\* Part of a thesis for the degree of D.Sc. of the University of Edinburgh.



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(PLATE XVIII)

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portal tide which varied with the alimentary absorption. The capsulo-trabecular system showed predominant changes with obstruction of the splenic vein, while portal vein occlusion was followed by distension of the sinuses. He held that the morbid picture of the "fibro-adenoma" of Banti could be induced merely by congestion. Analysis of his experimental work brings out the important fact that in no instance was there any evidence of definite proliferative reactions or gross splenomegaly.

In order to determine whether proliferative reactions could be induced in the spleen by portal congestion it was necessary to study the effects at different stages of portal obstruction.

#### *Material and methods*

The following methods of inducing portal obstruction in rats and rabbits were employed: (1) complete closure of the portal vein at the gastro-hepatic omentum by a silk ligature, (2) partial obstruction of one-third to two-thirds of the diameter of the vein by a small elastic band or loop of silk thread, (3) partial obstruction of the vein by a kink produced by a loop attached to the under surface of the liver, (4) obstruction of the left main branch by a ligature close to the *porta hepatis*, (5) partial obstruction of the vein by means of a pituitary clip clamped on one side of the vein so as to induce various degrees of constriction, (6) obstruction by experimentally induced portal phlebitis and phlebosclerosis by the action of caustics on the portal vein.

The portal vein of the rabbit is peculiar in that it is continued to the under surface of the liver as the main left branch while a smaller right branch is given off before reaching the liver. This supplies the right posterior and caudate lobes. The right anterior and the left anterior and posterior lobes, together forming the main bulk of the liver, are supplied by the main left branch. The common bile duct lies immediately on the ventral surface of the portal vein a little to the right, with the hepatic artery deeper down and more to the right of the vein. In the rat the vein is very thin and small but otherwise the relations are similar.

Under ether anaesthesia the abdomen was opened by a median incision extending vertically downwards from the xiphoid cartilage for an inch and a half. The anterior margin of the liver was kept up by means of a retractor and the lower border of the stomach was pulled out to bring out the gastro-hepatic omentum. A curved director was then passed from behind forwards through the omental fold to include the vein without tearing through the wall. The hepatic artery and common bile duct were gently dissected off by a blunt needle and a loop of the ligature passed through and dealt with in one of the ways mentioned above. For ligating the main left branch it was necessary to ligature high up close to the under surface of the liver, beyond the smaller right branch. When a pituitary clip was used the operation of partial clamping was easily carried out, since the clip could be slipped on from the right side over the omental fold, care being taken to see that the hepatic artery and common bile duct were not included. The procedure was facilitated by the use of special clip forceps designed by Mr Norman Dott for pituitary operations. It was found on subsequent operation that, if the clip was well clamped down, there was no danger of its slipping. The abdominal wound was sutured in two layers.

Animals which survived the operation were killed after periods ranging from 2½ to 6 months. The spleen was removed entire with the splenic vessels and measured and weighed before fixation. In most cases, both spleen and liver were histologically examined. After fixation in Helly's

findings in sections were stained by (1) Mayer's hemalum and eosin, (2) Azan iron fastatoxylin and van Gieson's stain, (3) Heidenhain's iron-haematoxylin and (4) Wilder's modification of the Loos-Bielchowsky stain for reticulum.

## RESULTS

### *General effects*

Some of the other animals died of hæmorrhage from juncture of the thrombosed vein when the director was passed from behind. Only one rabbit developed sepsis. This, however, was quite superficial and due to infection of the suture material. In all the surviving animals portal congestion was well defined even six months after as judged by the condition of the splenic veins at autopsy as well as by the state of the trabecular and pulp veins in histological sections.

### *The effect of complete portal obstruction*

Complete ligation of the portal vein in rats and rabbits was invariably fatal in from 1 to 48 hours in rats, in from 20 hours to 4 days in rabbits (table, p. 360). Death was due to mesenteric thrombosis, followed by rupture of the venous tributaries into the intestine and fatal hæmorrhage. The post mortem appearances were remarkably constant. The mesenteric veins below the ligature were congested, the splenic vein distended and swollen. The coils of the jejunum and the upper coils of the ileum were dark purple in colour and the smaller venous twigs of the mesentery and in the subserous coat of the intestine were all turgid. The lumen of the intestine was filled with clotted blood. The spleen was enlarged to about three times the normal size. It was dark purple in colour, the capsule was tense, and on section the cut surface oozed blood. The liver was generally pale and flabby but no other changes could be demonstrated.

Histologically the changes in the spleen varied only in degree. In rats dying 3-12 hours after the operation the condition of the spleen resembled that met with in venous infarction. The capsule appeared as an attenuated layer of fibrous tissue which had lost all wavy wrinkling and looked like the distended wall of a bladder, the trabeculae were very thin and trabecular branching was almost indistinguishable except by the azan stain. The congestion was so marked that the cytoplasmic reticular synovium was almost completely obscured and only the attenuated remnants of a mesh-work could be made out by van Gieson's and Heidenhain's azan stains. The protoplasm of the pulp synovium had disappeared in many places and only a few drawn-out threads remained. Where hæmorrhages had occurred, the synovium had undergone necrosis, a change reminiscent of the venous infarction met with in the cerebral cortex following thrombosis of the cortical veins. Owing

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Under ether anaesthesia the abdomen was opened by a median incision extending vertically downwards from the xiphoid cartilage for an inch and a half. The anterior margin of the liver was kept up by means of a retractor and the lower border of the stomach was pulled out to bring out the gastro-hepatic omentum. A curved director was then passed from behind forwards through the omental fold to include the vein without tearing through the wall. The hepatic artery and common bile duct were gently dissected off by a blunt needle and a loop of the ligature passed through and dealt with in one of the ways mentioned above. For ligating the main left branch it was necessary to ligature high up close to the under surface of the liver, beyond the smaller right branch. When a pituitary clip was used the operation of partial clamping was easily carried out, since the clip could be slipped on from the right side over the omental fold, care being taken to see that the hepatic artery and common bile duct were not included. The procedure was facilitated by the use of special clip forceps designed by Mr Norman Dott for pituitary operations. It was found on subsequent operation that, if the clip was well clamped down, there was no danger of its slipping. The abdominal wound was sutured in two layers.

Animals which survived the operation were killed after periods ranging from 2½ to 6 months. The spleen was removed entire with the splenic vessels and measured and weighed before fixation. In most cases, both spleen and liver were histologically examined. After fixation in Helly's

to the distension of the pulp mesh with blood, only a few groups of cellular clusters could be made out. Some of these were the compressed malpighian follicles, others were outlying islands of lymphoid tissue which are normally present in the rat's spleen. The malpighian follicles had retained their basic structure, but the three well defined zones normally present in the rat's spleen were all compressed into one cluster of lymphoid cells, while the lymphoid reticulum was hardly visible. On the whole the appearance of the spleen was that of a few islands of lymphoid tissue lying in a mass of blood which had not coagulated, but had retained to a great extent the configuration of the spleen owing to the presence of a thinned out fine work of reticular mesh (fig. 1). The sinuses, trabecular veins and pulp veins could not be distinguished except by special staining. At the extreme periphery of the lobule the sinuses appeared as distended sacs.

In rats and rabbits dying 24-48 hours after operation the congestive reaction was still marked. The engorgement was subcapsular and peritrabecular forming a distinct zone involving the periphery of the splenic lobule and leaving the malpighian follicles comparatively free. These were compressed, however, by the distension with blood beyond the marginal zone, where the arterial capillaries open out into the reticular mesh. In places, a few red blood cells had percolated into the follicles. The secondary lymphoid foci in the rat's spleen appeared compressed and grouped together by the congestion of the pulp. The reticular synectium was distinct, but the meshwork was irregular and appeared broken up and the number of nuclei was reduced. Here and there hemorrhage had taken place.

### *The effect of partial obstruction*

In rats after partial obstruction of 2½ months' duration induced by an elastic ligature, the spleen had undergone considerable shrinkage as compared with the acute stage of congestion seen at laparotomy one week after operation. The capsule was slightly more opaque than normal and the whole organ had a lobulated appearance. Microscopically congestive changes were quite well defined. Sinus engorgement was most marked in the subcapsular and peritrabecular zones. In the perimalpighian zone there was a diffuse engorgement of the pulp mesh. The capsule and trabeculae were thickened and had caused irregular contraction of the organ. The malpighian follicles appeared unaffected except for a slight increase in the reticular mesh. Azan staining showed slight fibrillary increase in the pulp.

In rabbits killed from 4½-6 months after operation there was little splenic enlargement (table). The capsule was generally



## FALL HISTOLOGICAL OBSERVATIONS



FIG. 1.—Rabbit spleen 24 hours after partial ligation of portal vein. The appearance of the spleen is that of a whole pulp and of blood with the venous mesh visible. Malpighian follicles and focal lymphoid clusters compressed and disintegrated. H and E.  $\times 120$ .

FIG. 2.—Rabbit spleen 6 months after partial ligation of portal vein. Note congestion of sinuses and pulp veins, hemorrhages and trabecular thickening. H and E.  $\times 120$ .

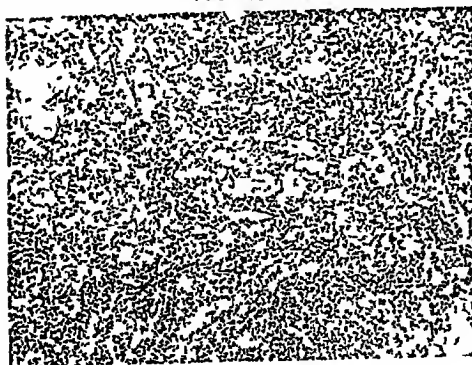


FIG. 3.—Rabbit spleen showing dilatation of sinuses after chronic congestion of 6 months duration. Andorson's hematoxylin and van Gieson.  $\times 95$ .



thickened and more opaque than normal. In some spleens an irregular lobulation was seen on the surface. Histologically, venous congestion was indicated by dilatation of the pulp veins and the sinuses at the periphery of the lobule (figs 2 and 3). The trabecular veins were markedly engorged. There was an increase in the fibrillary reticulum in the pulp around the dilated sinuses and pulp veins. Thickening of the capsule and trabeculae was quite distinct, and spread of fibrils from the trabeculae into the surrounding pulp could occasionally be made out. Malpighian follicles were variable in size, some had undergone atrophy, others were unaffected. An increase of the lymphoid reticulum of the follicle could be made out, but this seemed to be related to a slight general increase in the fibrillary meshwork rather than any definite spread from the arterioles. On the whole the appearance was suggestive of a fibroid atrophy which was compensated by the state of distension of the sinuses. Proliferative changes were absent in the pulp cords and malpighian follicles. The appearance of lobulation was due to contraction of the trabeculae.

In rabbits with partial obstruction induced by clipping, one developed thrombosis of the portal vein and died two weeks after the operation. The spleen showed venous infarction. In the other two the congestive reaction was quite marked two weeks and one month after operation, indicating that some degree of portal stasis had developed. The histological changes were similar to those induced by partial obstruction by ligation, but capsulo-trabecular thickening and fibrosis had not commenced.

In two rats an attempt was made to induce partial obstruction by passing a loop round the portal vein and tying it up to the under surface of the liver. However, both animals developed portal thrombosis and died from hæmorrhage into the intestine. The condition of the spleen was one of venous infarction.

Liver changes were slight and inconstant in both rats and rabbits.

#### *The effect of portal phlebitis.*

Portal phlebitis was induced in 2 rats by cauterising the vein with pure carbolic acid and a strong solution of iodine respectively. Four months later, the under surface of the liver and the omentum had become matted together to form a thick irregular mass in which the portal vessels were embedded. There was little splenic enlargement. Histologically the spleen showed marked capsulo-trabecular thickening, with engorgement of the sinuses, pulp veins and trabecular veins. The malpighian follicles showed little alteration. Fibrillary increase was little marked, nor were there any changes in the malpighian follicles suggestive of "fibro-adenic". The liver showed portal tract infiltration with mononuclear cells and lympho-

## EXPERIMENTAL SPLENOHISTIOLOGY



FIG. 1—Rabbit spleen 12 hours after congestion of portal system. The appearance of sinusoids in the whole pulp area of blood with little syncytial meshwork. Malpighian follicles and focal lymphoid clusters comprise islands isolated from the pulp. H and E  $\times 120$ .

FIG. 2—Rabbit spleen 6 months after partial ligation of portal system. Note congestion of sinusoids and pulp cells hemorrhages and trabecular thickening. H and E  $\times 120$ .

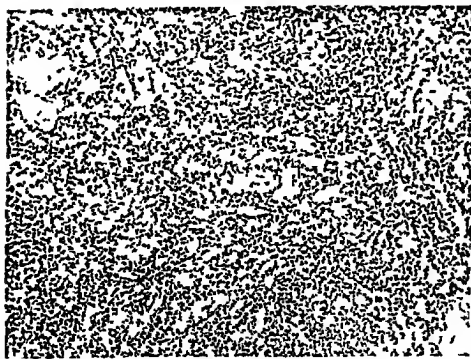


FIG. 3—Rabbit spleen showing dilatation of sinusoids after chronic congestion of 6 months' duration. Androsann's hematoxylin and van Gieson  $\times 95$ .





which Gauckler (1905) described as a "sclérose hypertrophique pulpaire" that characterises splenomegalic cirrhosis. The post-congestive fibrosis resembles more the "sclérose atrophique" reaction and ends in cyanotic atrophy rather than hypertrophy. Hueck has argued that in peripheral stasis as in obstruction to the splenic and portal veins the effect of the collateral circulation would be to render the congestion intermittent and so cause hypertrophic changes. The experimental work of Wicklem and Warthun on obstruction to the splenic veins as well as the present study of the effects of portal obstruction shows that venous congestion in the spleen of whatever type is followed by an immediate and progressive enlargement which lasts only for a short time and is gradually followed by shrinkage and fibrosis. Hyperplastic reactions do not play any part in the experimental animal after portal obstruction. On the other hand the later shrinkage of the spleen seems to be more related to the gradual atrophy of the pulp from compression of the syncytium by the distended vessels and the erythrocyte accumulations in the pulp mesh. A gradual fibrillary increase would also favour shrinkage while the turgidity of veins and sinuses and the rigidity of their walls would tend to counteract this factor. These experiments therefore do not support the view that the gross splenomegaly and hyperplastic changes which characterise the Banti syndrome are due to a mechanical block in the portal circulation. Some other factor is essential to induce proliferative changes in the spleen.

#### SUMMARY.

1 Portal obstruction in rats and rabbits has been induced by various operative measures having the effect of narrowing the lumen of the portal vein.

2 After complete occlusion of the vein there is a splenic enlargement of twice or three times the normal and no more, the condition of the spleen is one of venous infarction.

3 After partial obstruction of 3-6 months' duration there is very little splenic enlargement. The spleen shows dilatation of the sinuses, distension of the pulp and trabecular veins, and a variable atrophy of the pulp with slight fibrillary increase. Hyperplastic reactions are absent.

4 These experiments do not support, but seem to negative the view that the Banti syndrome is due to blockage of the portal vein.

The author's thanks are due to Professor Murray Drennan for his kind criticism and to Dr J. R. Cameron for assistance in the use of pituitary clips in two of these operations. The work was supported by a grant from the Earl of Moray Fund of Edinburgh University.













Colonies about 1 mm. in diameter are produced on the surface of agar or blood agar plates. On the latter the zone of hæmolysis is well marked on first isolation from an infected animal, but gradually diminishes in subculture, being eventually reduced to about half its original dimensions. The colonies can be distinguished from those of pneumococci by the absence of autolysis and from those of hæmolytic streptococci recently isolated from human infections by their slightly larger size, as well as by the fact that on repeated subculture they show no tendency to differentiate into rough and smooth forms (fig. 4). Cultures are easily kept alive and virulent when subcultured at weekly intervals on agar slopes and in tubes of broth alternately. The organism shows a remarkable tendency to grow less abundantly and eventually to die out when maintained either in fluid or solid medium alone. After 104 passages the morphological and cultural characters were unchanged and the organism was still virulent for mice. In the dried spleen of a mouse dead of the infection the organism can be kept alive and virulent for at least eighteen months.

#### *Fermentation reactions.*

These were tested in  $4 \times \frac{1}{2}$  inch tubes containing 5 c.c. of casein digest broth (glucose-free) with Andrade's indicator and 1 per cent. of all carbohydrates except glucose and dulcitol which were used in concentrations of 0.5 per cent. The tubes were inoculated with a loopful of a 12 hours' culture from an agar slope and incubated at 37° C. for six days, when final readings were made. Good growth took place in all tubes overnight. The final pH in glucose broth was determined by the use of appropriate indicators (bromphenol blue and chlorphenol red) and buffer solutions of known pH. Hydrolysis of sodium hippurate was tested for by the technique of Ayer and Rupp (1922).

Acid (without gas) was produced in glucose, lactose, sucrose, trehalose and salicin, while there was no fermentation of mannitol, dulcitol, sorbitol or inulin. The final pH in glucose broth was 3.8. Sodium hippurate was hydrolysed.

#### *Hæmolysin production*

The production of hæmolysin in fluid culture was investigated by the procedure of McLeod (1914-15) and Channon and McLeod (1929). It was found that the maximum amount of hæmolysin was present in a culture after 12-18 hours' incubation (table 1). This point is chiefly significant in relation to a finding reported by Griffith in his study of the Neufeld strain. He noted that the Neufeld and Wamoscher strains were identical in their serological characters and high virulence for mice. The only difference between them was that colonies of the Wamoscher strain caused the larger zone of lysis on a blood agar plate. Regarding the production of

hæmolysin in fluid media by the Neufeld strain he states, "A soluble hæmolysin is formed in broth cultures of Aronson N incubated for 24 hours, its activity is increased if the culture is allowed to stand at room temperature for 3 days" He does not give details of method. This finding is in marked contrast to those recorded in table I and suggests that the two strains are not in all respects identical.

TABLE I

*Hæmolysin titre of culture in 20 per cent ox serum broth at different periods of growth*

Period of incubation	Minimal hæmolytic dose
6 hours at 37° C	0.1 c.c.
0	0.05 "
12	0.025 "
15	0.01
18	0.01
21	0.1 "
24	0.1
24 hours at 37° + 24 hours at 22° C	0.1
24 hours at 37° + 48 hours at 22° C	Hæmolysis of about 25 per cent of cells with 0.25 c.c.

Minimal hæmolytic dose = smallest amount of culture required to produce complete lysis of 0.5 c.c. of a 3 per cent suspension of red blood cells in 2 hours at 37° C.

*Diffusibility of the hæmolysin* Hæmolysin produced by this streptococcus differs in one important particular also from that produced by strains of hæmolytic streptococci which have been isolated from human lesions. As noted by McLeod and many others such streptococci secrete a hæmolysin which is very diffusible and readily filterable. Only Lyall (1914) has recorded any difficulty in separating hæmolysin from organisms. He tested only one of his strains. Cowan (1922) found that cultures which grew with much deposit of the organisms at the foot of the tube required to be well shaken before the hæmolysin was obtained apart from the organisms. Actively hæmolytic cultures of Aronson's streptococcus have been repeatedly centrifuged at different speeds and for varying lengths of time. On no occasion has the supernatant fluid been found to contain more than one tenth of the amount of hæmolysin found in the whole culture. If, however, the organisms in the centrifuged deposit were shaken up with fresh broth or serum broth, the suspension was almost as actively hæmolytic as the original (table II). The result was different if saline or peptone water was used to re-suspend the organisms. A suspension of organisms from the surface of an agar slope culture in broth or serum broth was not hæmolytic. Three cultures of hæmolytic streptococci recently isolated from human lesions were tested in the

same way. In all of them a considerable fraction of hæmolysin was obtained without difficulty in the supernatant fluid (table II)

TABLE II.

*Distribution of hæmolysin in centrifuged cultures of (A) Aronson's streptococcus and (B) Streptococcus pyogenes from human infection.*

Test material	Minimal hæmolytic dose
Whole culture (A)	0.01 c.c.
Supernatant fluid (A)	Lysis of about 25 per cent. of cells with 0.25 c.c.
Organisms suspended in serum broth (A)	0.025 c.c.
Organisms suspended in saline (A)	Lysis of about 25 per cent. of cells with 0.1 c.c.
Whole culture (B)	0.025 c.c.
Supernatant fluid (B)	0.025 „

These results show that the hæmolysin produced in fluid cultures of Aronson's streptococcus is less diffusible than that produced by a typical hæmolytic streptococcus from a human lesion. Both hæmolysins are completely inactivated after heating for 15 minutes at 56° C.

### Virulence

*Cultures in serum media.* Young cultures 6-18 hours old grown in 20 per cent. ox serum broth always proved highly virulent for mice of 18-22 g. weight. Allowing for the difference in size of the animal they were at least as virulent for the rabbit. Injection of 1 c.c. of culture into the peritoneum of guinea-pigs produced no ill effects.

During a period of 18 months 87 mice were inoculated with amounts of serum broth cultures varying from 0.5 c.c. of undiluted culture to 0.25 c.c. of culture diluted 1:1000 million. Sixty-two of these mice received injections of 0.25 c.c. of culture diluted 1:1 million or smaller amounts. All these animals died in 18-48 hours with general septicæmia, whether the inoculation was subcutaneous or intraperitoneal. The number of organisms in the higher dilutions of culture was estimated on six occasions by plate methods, and it was found that one or two cocci were sufficient to produce a lethal effect. Death resulted as rapidly from such small doses as after injection of larger amounts of culture.

*Cultures in serum-free media.* Aronson's streptococcus differs from hæmolytic streptococci from human infections in that it remains virulent for mice even when subcultured frequently in media which contain no serum. In the present investigation the strain was passed alternately through broth and agar without serum at intervals of 5-7 days. It was first used in 1929 and is still virulent for mice after nearly five years. From October 1932 to August 1934 it was

subcultured 104 times on ordinary agar and broth without serum, from September 1934 to October 1935 it was kept in boiled blood broth with three subcultures, from October 1935 to July 1937 it was once more passed at about weekly intervals (90 subcultures) through broth and agar without serum. Although still lethal for mice, it is not so highly virulent as serum cultures. Intraperitoneal injection of 0.25 c.c. of a 1:1000 dilution of culture without serum is still sufficient to kill mice in 24-48 hours but smaller amounts of culture are less regular in their effects. Even after shorter periods, cultures which are kept in media without serum become less virulent than cultures kept in dried spleens or in media with serum, although their full degree of virulence can be restored by mouse passage (three such passages are sufficient) or culture in media containing serum. During a variable period, however, cultures in media without serum do retain full virulence.

*Grading of virulence.* As examples of the difficulty which attends the use of the term "minimal lethal dose" in relation to bacterial cultures the following may be quoted.

On 27.10.32 a culture was made from the heart blood of an infected mouse and kept thereafter on broth and agar without serum. Between 27.10.32 and 10.4.33 twenty-eight subcultures were made and virulence titrations (see below) carried out with eleven of them, nos. 2, 6, 7, 9, 15, 19, 20, 21,

TABLE III

*Virulence of Aronson's streptococcus in media containing no serum*

Subculture		Mice		Dilution of culture
No.	Date	Inoculated	Died	
2	20.11.33	1	1	1:500 × 10 <sup>6</sup>
		3	3	1:5000 × 10 <sup>6</sup>
10	8.1.34	1	1	1:50 × 10 <sup>6</sup>
		1	1	1:500 × 10 <sup>6</sup>
		1	1	1:5000 × 10 <sup>6</sup>
17	16.1.34	1	1	1:100 × 10 <sup>6</sup>
		1	0	1:500 × 10 <sup>6</sup>
		1	1	1:5000 × 10 <sup>6</sup>
18	22.1.34	7	2	1:500 × 10 <sup>6</sup>
10	1.2.34	5	2	1:5 × 10 <sup>6</sup>
22	8.2.34	4	4	1:1000
		3	3	1:25 000
		3	1	1:500 000
24	19.2.34	3	2	1:10 000
		3	2	1:30 000
		3	2	1:100 000
		3	1	1:180 000
		3	3	1:300 000

24, 25, 28) Thirty-five mice received intraperitoneal injections of 0.25 c.c. of high dilutions (1:500,000–1:5000 million) and all died of streptococcal septicæmia in less than 48 hours. Similarly between 20.11.33 and 8.1.34 the organism, transferred at intervals of 3–4 days, retained full virulence for 16 subcultures in media without serum. Subcultures 17, 18, 19, 22 and 24, however, were of a lower degree of virulence (table III). This change in virulence was not accompanied by any alteration in morphology, colony form, fermentation reactions or capacity to produce hæmolysin.

It can be seen from table III that with cultures of less than full virulence the natural resistance of the experimental animal plays a decisive part in determining the outcome of experimental infection. An even more striking illustration is that in table IV,

TABLE IV  
*Irregular effects produced by cultures of intermediate virulence*

Mouse	Culture dilution	Time of death
1	Undiluted	24 hours
2	1:10	24 "
3	1:100	48 "
4	1:1,000	48 "
5	1:10,000	5 days
6	1:100,000	S
7	1:500,000	48 hours
8	1:500,000	48 "
9	1:1 × 10 <sup>8</sup>	S
10	1:5 × 10 <sup>8</sup>	48 hours
11	1:500 × 10 <sup>6</sup>	S
12	1:500 × 10 <sup>6</sup>	48 hours

S = survived for 2 months

where it is shown that mouse 6 survived intraperitoneal inoculation of 5000 times the lethal dose for mouse 12. In this case the strain had been subcultured 57 times on media without serum over a period of 8 months.

It is therefore clear that with cultures of less than full virulence the term "minimal lethal dose" becomes impossible of definition owing to the uncertainty of the result of inoculating a given amount of culture. Even with cultures of full virulence the true meaning is not easy to define, since in these cultures one or two organisms produce a lethal effect and the error in sampling from such high dilutions is necessarily considerable. To overcome this difficulty Trevan (1927) has urged the use of the term "*average lethal dose*" in place of "*minimal lethal dose*". "*Average lethal dose*" he defines as the smallest amount of the toxic material which will kill 50 per cent of the test animals within a stated time. Morgan and Petrie (1933), in testing the efficacy of anti-pneumococcal serum in the protection of mice against pneumococcal cultures, found it necessary to inoculate anything from 99–200 mice with each single dilution of culture in order to satisfy themselves that their results were statistically significant. While the use of large numbers of test animals is necessary to obtain statistically accurate results, it is frequently obvious that one culture is much more virulent than another and it is desirable to express this without undertaking investigations on so

large a scale. In the present work virulence has been graded roughly by the use of 10 mice for each culture. Tenfold dilutions were made ranging from undiluted culture to 1:1000 million, and 0.25 c.c. of each dilution was inoculated into the peritoneal cavity of one mouse. In this way four grades of virulence have been defined, as follows:

**Full virulence.** All the animals die within 3 days of inoculation.

**Intermediate virulence.** Animals which receive undiluted culture and dilutions 1:10 and 1:100 die within 3 days, but some of those which receive weaker dilutions either survive or die after 3 days.

**Low virulence.** Undiluted culture and dilutions 1:10 and 1:100 are uncertain in their lethal effect.

**Avirulent.** All mice survive. No culture has been called avirulent unless it has been shown that intraperitoneal inoculation with 0.5 and 0.25 c.c. of undiluted culture failed to kill at least six mice.

While this grading is necessarily somewhat arbitrary it distinguishes cultures which are certain in their lethal effects from those which are uncertain. Employing these terms the virulence of Aronson's streptococcus may be summarised as follows:

Cultures in serum broth possess and retain full virulence. Cultures in media without serum retain full virulence for a variable period (3-6 months) and thereafter acquire intermediate virulence, but no further fall in virulence results even up to two years. Such cultures are morphologically and culturally indistinguishable from those of full virulence.

#### *Induced variations in Aronson's streptococcus*

Among streptococci isolated from human infections, variations of colony form occur, as a rule, spontaneously and cultures can be readily dissected into rough (R) and smooth (S) variants of different virulence by selection of colonies alone (Cowan, 1922, 1923, 1924, Todd, 1927, 1928, Eagles, 1928, Andrewes, 1928, Andrewes and Christie, 1932). In a given culture either the R form is virulent and the S avirulent or *vice versa*. According to Andrewes and Christie the R forms of streptococci are more specific in their agglutination reactions than the S forms. Numerous attempts were made by selection alone to obtain an R variant of Aronson's streptococcus from the S colonies, but the organism was found to differ from other streptococci in that it could not be dissociated in this way. It appeared of importance therefore to determine whether procedures similar to those used to obtain R pneumococci would produce R variants. Nakamura exposed cultures of a virulent streptococcus to the action *in vitro* of a 1:25,000 solution of trypanflavin for periods of  $\frac{1}{2}$ -1 hour and found that this procedure decreased the virulence for mice. Nakamura used "hochvirulent Streptococcus Arensen" but gave no other particulars of the culture either before or after treatment with trypanflavin.



*Technique* A batch of ordinary nutrient broth and agar was prepared in the usual way. One-tenth of this was set aside for control. The remainder was used to prepare media containing various concentrations of acriflavine ranging from 1:12,500 to 1:1 million by the addition of suitable quantities of a 1:1000 solution. The media thus prepared were tubed (broth about 10 c.c., agar about 5 c.c.) and sterilised in the autoclave. A young agar culture was used to inoculate the entire range. Growth resulted only in the media containing 1:1,000,000 acriflavine. This growth was subcultured into the entire range and growth now resulted in 1:800,000 acriflavine broth. From this, and on subsequent occasions from the highest concentration in which growth occurred, subcultures were made at intervals of a few days. The organism was thus induced to adapt itself gradually to increasing amounts of the antiseptic. Transfers for this purpose were made alternately from broth to agar and from agar to broth and a control culture was maintained on the same media without acriflavine. No serum was used in any of these cultures. In the higher concentrations growth was often slow at first and large inocula were necessary. Finally the organism was grown without difficulty in either broth or agar containing 1:25,000 acriflavine. Nineteen subcultures were required in each instance to carry the culture through the various concentrations to that of 1:25,000. Tolerance to the antiseptic had thus increased forty times. The organism was maintained for 65 cultures (9 months) in 1:25,000 acriflavine and in another series for 110 subcultures (2 years) in the same medium. It could never be grown in media containing 1:12,500 acriflavine.

Films of each subculture were examined by the usual stains as well as by the eosin method for capsules. Cultures were plated on ordinary agar and on blood agar and the colonies examined under the low power. It was found that as the organism became adapted to growth in the lower concentrations of acriflavine (1:1,000,000–1:250,000) numerous colonies with crenated edges appeared. When subcultured in media without acriflavine, however, these reverted rapidly to the appearance of S colonies in the control culture. In the higher concentrations (1:100,000–1:25,000) alterations in colony appearance became more marked. Colonies appeared which were definitely rough. On plating, however, they gave rise to both smooth (S) and rough (R) forms (figs 4 and 5). In broth culture they frequently showed a mixture of capsulated and non-capsulated organisms (fig 3). The length of chain increased and the growth in broth was chiefly at the bottom of the tube in contrast to the even turbidity produced by the control culture. By repeated selective subculture with picked R colonies growths were obtained in which all the colonies were rough and of which films showed only very long-chained forms without capsules (fig 2). Growth took place entirely at the bottom of broth tubes leaving a clear supernatant fluid. This procedure of producing an acriflavine-resistant strain and deriving from it an R form by selective subculture was repeated on three separate occasions.

*Virulence of the R variant* The R variant when freed in this way from all S forms was non-virulent. A pure R variant in a

## ARONSON'S STAIN TECHNIQUE

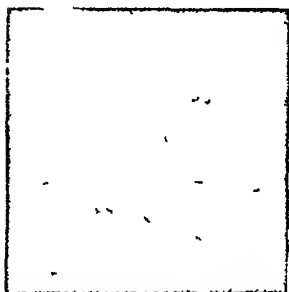


FIG 1—S form 24 hour broth culture Fovsin stain for capsules  $\times 1000$



FIG 2—R variant 24 hour broth culture Fovsin stain for capsules  $\times 1000$



FIG 3—R variant 24 hour broth culture Early stage of reversion to S form Fovsin stain for capsules  $\times 1000$

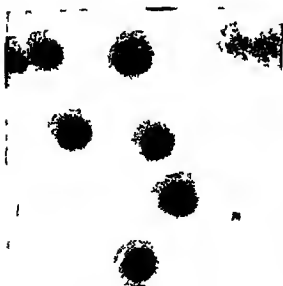


FIG 4—S colonies on agar plate Transmitted light  $\times 20$



FIG 5—R colonies on agar plate Transmitted light  $\times 20$



dose of 0.25 cc of undiluted culture was inoculated intraperitoneally into 15 mice, 7 different subcultures being tested. 22 mice received intraperitoneal inoculations of 0.25 cc of the same subcultures in dilutions ranging from 1:10 to 1:1 million, all survived. Another pure variant was inoculated over a period of 5½ months into a total of 46 mice. All the animals were given 0.25 cc of the undiluted culture into the peritoneal cavity and all survived. It is therefore clear that when grown in media with 1:25,000 acriflavine Aronson's streptococcus gives rise to an R form similar in its avirulence, loss of capsule and altered colony form to that shown by other workers to arise from pneumococcal cultures under the influence of an inhibitory agent.

Essentially similar results have been recorded by Griffith with the Neufeld strain. He obtained his R variant by a combination of three procedures—growth in homologous antiserum, growth at 40° C and selective subculture.

*Stability of R variant.* Apparently one stable R form has been produced, since it has now been cultivated for over two years on ordinary agar and broth with monthly subcultures and its form has not altered. On the other hand the first R variant obtained, which appeared to have been completely purified of S forms after 10 selective subcultures, showed, six months later, at the 24th subculture, the presence of a few capsules (fig. 3) in films from a broth culture, and after three further subcultures without selection this culture became almost completely S in type and returned to intermediate virulence. Eventually it reverted to a purely S culture, but virulence never became full, media without serum being used. The tendency of the culture to grow in the S form was further illustrated by the behaviour of a culture in 1:25,000 acriflavine which was never subcultured by selection of colonies. After 65 subcultures in 1:25,000 acriflavine broth and agar alternately, it showed on plating a ratio of R to S colonies of about 9 to 1. It was avirulent, as 14 mice survived 0.5 cc of whole culture intraperitoneally. At the 84th subculture in 1:25,000 acriflavine, however, the culture was almost entirely S in type and was found to have regained virulence of a low grade, since 5 out of 7 mice died after intraperitoneal inoculation with 0.25 cc of undiluted culture. The remarkable natural stability of the virulence of this organism is emphasised by this tendency of artificially produced avirulent cultures to revert spontaneously toward the grade of virulence possessed by ordinary stock strains on artificial media. At one end of the scale are the fully virulent passaged cultures, at the other the cultures artificially made and kept avirulent. Between these extremes is an intermediate range of virulence to which all forms tend to revert unless special measures are adopted to prevent it.

*Antigenic relationship of S and R forms* Antisera against both S and R forms were obtained by repeated intravenous injection of killed suspensions into rabbits. For agglutination tests the organisms were grown on casein agar slopes for 24 hours at 37° C. The growths were then washed off in 0.45 per cent NaCl. With the R organisms it was necessary to employ this concentration of NaCl in order to prevent spontaneous agglutination and it was also used for the S organisms although they did not spontaneously agglutinate in 0.85 per cent NaCl. The results were read after 4 hours at 56° C.

The S antiserum agglutinated homologous S organisms to a titre of 1/160 and R organisms to a titre of 1/40. The R antiserum agglutinated homologous R organisms to a titre of 1/40 but did not agglutinate S organisms at all. No agglutinin absorption tests were carried out. Results similar to these are reported by Griffith with the Neufeld strain.

*Hæmolysin in R cultures* The R avirulent organisms produced in serum broth cultures the same amount of hæmolysin as the S organisms of full and intermediate virulence. On rabbit blood agar plates the two types of colony produced equally large zones of clearing. With this streptococcus therefore there is no correlation between ability to produce hæmolysin and virulence for mice. Lancefield (1934b) reported that a variant of her streptococcus O 90—the Wamoscher strain—lost the power to produce hæmolysin or pigment and yet retained virulence.

Both R and S strains had the same fermentation reactions, both were killed by exposure to 60° C for 10 minutes and both were insoluble in bile.

### Summary

1. The results of a study of the Wamoscher strain of Aronson's streptococcus from the Koch Institute, Berlin, are recorded.
2. The hæmolysin produced by this organism appears to be less diffusible than that of hæmolytic streptococci from human infections.
3. By adaptation to growth in acriflavine the natural stability of this organism in respect of virulence and certain other properties has been overcome and R variants have been produced.
4. These variants are avirulent and non-capsulated and they produce rough colonies. They are serologically but not biochemically distinct from the original S forms, to which they show a marked tendency to revert in the earlier stages of adaptation.

The photomicrographs are, I wish to express my thanks, as well as who kindly supplied the org

John Kirkpatrick to whom I wish to express my thanks, as well as the Koch Institute, Berlin,

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## SHORT ARTICLES

616—006 452 618 146

### TWO CASES OF MALIGNANT MUCUS SECRETING CYSTADENOMA OF THE CERVIX UTERI

MARY A. GRIFFIN

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(PLATES XX AND XXI)

Adenocarcinoma of the cervix uteri is much less common than the squamous type though the incidence in the reports of different laboratories varies greatly. Norris (1936), reviewing the statistics from all countries between 1919 and 1930, analysed 9509 cases of cervical cancer and obtained an average of 5.7 per cent of glandular adenocarcinoma. His own material yielded 8.45 per cent over 508 cases (1900 to 1934), and the lowest figures were those from the Freiburg Clinic (Keller, 1930)—1.62 per cent of 310 cases from 1915 to 1925. The highest percentage was that of Bartlett and Smith (1931) at the Free Hospital for Women, Boston—11.7 per cent in 560 cases between 1875 and 1920, while Ross in the Mayo Clinic (1922) found 10.1 per cent in 277 cases.

In advanced cases of carcinoma of the cervix it is sometimes difficult to determine whether the growth is of glandular or squamous origin, since glandular carcinoma may give rise to a solid alveolar type of growth. Martzloff (1928), in a series of 70 cases, showed that in a third of these biopsy material was insufficient for classification and to this he attributed the great variation in the percentages published by different workers.

During the past 27 years, 749 cases of carcinoma of the cervix have been examined histologically at the Royal Samaritan Hospital for Women, Glasgow, 94 of these were of the glandular type (12.6 per cent) a proportion somewhat higher than that recorded in any of the series quoted by Norris. Among these were two which differed strikingly from the remainder in their histological characters, having the structure of malignant mucus secreting cystadenomata. In both cases the tumours were inoperable when the patients first came to hospital, in one a post mortem examination was subsequently performed.

#### Case I

*Clinical history.* The patient, a woman of 34, had been married for twelve years and had had 5 children, the last born in December 1933, all were normal spontaneous labours. Puberty occurred at the age of 15 and since then menstruation had been regular ( $\frac{34}{28}$ ) days, and her general health was good. At the end of April 1936 she began to suffer from a thick mucoid vaginal discharge which continued until her admission in August. The last regular period was six weeks before admission and was followed



by irregular profuse vaginal bleeding at intervals of one or two weeks, although the periods had previously been rather scanty. On admission there was considerable œdema of the legs of several days' duration, but the general condition seemed good.

Vaginal examination revealed a large soft growth infiltrating the cervix uteri and portions removed for histological examination had the structure of a mucus-secreting cystadenoma.

During most of her stay in hospital the temperature fluctuated between 101 and 103° F. On 7th September 1936 she had a rigor (105° F) and thereafter the swelling of the legs, which had become very marked on the right side, disappeared and the general condition improved. On 19th October, however, the right leg again became swollen and thereafter the general condition gradually deteriorated. Death occurred on 11th November 1936.

*Post-mortem findings* *Post mortem*, the cervix uteri was seen to be replaced by soft, translucent, gelatinous tumour tissue which had produced massive enlargement around its entire circumference and enormous elongation of the cervical canal which was distended with mucoid secretion. The growth extended into the vagina and the lower margin had a ragged surface where it had been curetted. The uterine body, normal in size, was displaced upwards by the enlarged cervix and at the internal os the tumour was seen to be invading the corporeal myometrium, the whole mass being fixed by infiltration of the paracervical tissues. The external, internal and common iliac lymph glands were invaded by new growth, and the lumbar glands extending up to the celiac axis were much enlarged, some measuring 1 x 2 ms. They were composed entirely of white tumour tissue and the surface was much congested. The tubes and ovaries showed no abnormality. The bladder could be separated from the vagina except at one small area where the tumour spread through its wall and projected into the vesical cavity as a number of soft sessile polypi on the posterior wall. The colon was distended above the uterus and constricted as it passed behind it, but was not invaded by the cervical growth. Septic infarcts were found in the spleen and in both kidneys. Both femoral veins were occluded by thrombus, but there was no histological evidence that this was associated with tumorous invasion of these vessels. The liver was enlarged and showed marked fatty change. Both lungs were œdematous. The heart, which was adipose, showed large, recent, friable vegetations on the mitral and aortic valves—a terminal endocarditis. One vegetation of the mitral valve had a white papillary appearance and was 1 cm in diameter. It had the usual histological characters. Metastases were not present in the liver and lungs.

*Microscopical examination* Portions of the tumour sent for microscopical examination on 4th August 1936 show a multilocular mucus-secreting cystadenoma (fig 1). The large cystic spaces are lined by high columnar cells and filled with mucoid material which is stained brilliantly by mucicarmine. The nuclei are situated basally and the inner (superficial) part of each cell contains mucus. Fine branching processes covered by these cells extend into the loculi, the appearances resembling those of pseudomucinous cystadenoma of the ovary. Inflammatory changes are present but the appearances at this time do not seem to indicate malignancy. Curettings taken on 20th August 1936 have a similar appearance, but a specimen obtained two months later (fig 2) shows a less papilliform structure, and areas are present where the epithelium has become cuboidal or flattened and there is evidence of stromal invasion, the appearances now being highly suggestive of malignancy. This was confirmed by material from the necropsy, portions from the region of the internal os and cervico-vaginal junction both showing invasion of the deeper muscular tissues. The enlarged lymphatic

## MALIGNANT CYSTADENOMA OF CERVIX



FIG. 1—Case I Section of tumour showing high columnar cells covering branching processes which project into the lumina of the cystic spaces  $\times 105$



FIG. 2—Case I Section of tumour showing cubical and columnar cells lining the gland spaces, with stromal invasion  $\times 270$

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glands from the posterior abdominal wall are replaced by glandular carcinoma in which the papillary architecture is not conspicuous (fig 3)

Sections through the bladder at the point of invasion show penetration of the muscular coat by mucus secreting glands. These have given rise to cystic nodules projecting from the submucosa into the lumen of the bladder and lined by tall columnar cells with numerous fine papilliform processes similar to those in the earlier curettings (fig 4)

## Case II

*Clinical history* The patient, a woman of 42, had been married for 21 years and had had 6 children and 3 miscarriages. Five months before admission to hospital on 9.11.33 she developed menorrhagia, metrorrhagia and vaginal discharge. There was no pain but the abdomen gradually increased in size, later, loss of weight and frequency of micturition were observed.

On vaginal examination a large cauliflower like cervical tumour was found measuring 3x2 ins. The cervical canal was obliterated and the growth was judged to be inoperable. A portion was removed for pathological examination. The microscopical appearances are similar to those seen in the first specimen from case I. Cystic spaces containing mucus and lined by columnar cells are present, and branching processes covered by these cells extend into the cavities of the cysts. In places malignant transition is noted, where the cells are more cuboidal and are invading the stroma.

The patient was readmitted on 2.12.33 and 50 mg of radium were inserted for five days. At this time 16 pints of fluid were removed from the abdomen and a drain was left *in situ*. A further portion of the tumour removed at this time shows unequivocal evidence of malignancy, the cells lining the glandular spaces being flattened or cuboidal while invasion of the stroma is apparent. The appearance is that of a malignant mucus secreting cystadenoma. Death occurred at home about six months later.

## Summary

Two cases of malignant mucus secreting cystadenoma of the cervix uteri are described. Both patients were multiparae and the disease occurred during the reproductive period. In one case death followed one year after, and in the other 7 months after the first onset of symptoms, among which menorrhagia, metrorrhagia and thick mucoid vaginal discharge were prominent features. In each case the neoplasm appears to have begun as a simple mucus secreting cystadenoma in which transition to malignancy followed, as is shown by extensive invasion of adjacent tissues and metastasis to the regional lymph nodes.

My thanks are due to Dr John Hewitt for permission to make use of the clinical records.

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# PRESERVATION OF COMPLEMENT FOR THE WASSERMANN REACTION.

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Complement, an essential reagent in the Wassermann test, is extremely unstable and rapidly loses its activity when kept under ordinary conditions even at 0-5° C. In this, as in most laboratories, it has been customary to use fresh guinea-pig serum as the source of complement for each set of Wassermann tests carried out in one day. This has obvious disadvantages. Samples of complement are variable in haemolytic power and fixability and this can only be obviated by the pooling of sera from several animals entailing considerable wastage as the volume of pooled serum is usually much in excess of the day's requirements. Various methods of preservation have been described, e.g. refrigeration of the serum at -15 to -20° C, desiccation of the serum *in vacuo* and storage in the dry form, the addition of glycerol, sodium chloride, sodium acetate, etc. The purpose of this note is to draw attention to a very simple yet effective method described by Sonnenschein in 1930 but apparently not well known in this country.

*Method* Fresh complement is obtained by severing the neck vessels of several guinea-pigs and collecting the blood. This is left overnight at 0° C and the serum separated. To a given volume of serum is added an equal volume of the following mixture.

Sodium acetate	12 g
Boric acid	4 g
Sterile distilled water	to 100 c.c.

The mixture is stored at 4° C.

Complement preserved by this method has been tested in parallel with fresh complement over a period of three years in this laboratory with satisfactory results and has now been adopted for the routine Wassermann tests. At first difficulty was experienced in that preserved complement stored in large bulk in a single container from which successive days' requirements were drawn was found to undergo progressive deterioration in haemolytic power. This was prevented by storing in each container a volume just in excess of that required for one day's tests. The value of the method can be appreciated by reference to the following details of preparation and use of one sample.

**Preserved complement no 6** This lot consisted of the pooled sera, total volume 156 c.c., from 20 guinea-pigs, the M.H.D. of the undiluted fresh serum being 0.008 c.c. An equal volume, i.e. 156 c.c., of the preserving mixture was added and the bulk then divided into 13 c.c. volumes in 24 vaccine bottles. A fresh bottle was opened each successive week for the routine Wassermann reactions, the M.H.D. being as follows.

Date	M.H.D. (c.c.)	Date	M.H.D. (c.c.)	Date	M.H.D. (c.c.)
17.2.37	0.008	14.4.37	0.008	9.6.37	0.008
24.2.37	0.007	21.4.37	0.009	16.6.37	0.007
3.3.37	0.006	28.4.37	0.007	23.6.37	0.007
10.3.37	0.007	5.5.37	0.006	10.8.37	0.007
17.3.37	0.006	13.5.37	0.008	18.8.37	0.007
24.3.37	0.007	19.5.37	0.008	25.8.37	0.007
3.4.37	0.008	26.5.37	0.008	1.9.37	0.008
7.4.37	0.008	2.6.37	0.009	8.9.37	0.008

In this instance there was no loss in hæmolytic power after 7 months. Several samples have now been tested after preservation for 12 months with equally satisfactory results. It will be noted that the M.H.D. of batch no. 6 was not constant but fluctuated about the mean 0.008 c.c. This was found to be due to variation in the ease with which the corpuscles in the hæmolytic system were lysed, as the M.H.D. of a number of preserved complements, tested together over a period of several weeks, showed a simultaneous rise or fall.

Apart from the advantage of securing uniformity of complement the method is exceedingly simple and is most economical in time and expense. Weekly killing is avoided, as is all wastage of serum. With preserved complement always available, the Wassermann reaction can be set up at short notice if urgently required. Further, small laboratories may now dispense with keeping guinea pigs specially for the supply of fresh complement and instead derive their supplies of preserved complement from some central source.

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## THE EFFECT OF SERUM MALTASE ON FERMENTATION TESTS WITH GONOCOCCI

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It has been the custom in these laboratories to use 5 per cent unheated horse serum nutrient agar as a substrate for testing the fermentation reactions of the Gram negative cocci. This medium appeared to be satisfactory for meningococci but with the gonococcus, using glucose, maltose and sucrose as test carbohydrates, one of two results was obtained. Either growth was absent or scanty and none of the three sugars was fermented, or growth was adequate but both glucose and maltose were fermented. The first result is not surprising, as horse serum agar is a poor substrate for the gonococcus. The second result, however, is irregular and has formed the subject of this investigation. As there was no acid production in the sucrose tubes, it seemed probable that the fermentation of maltose was due to preliminary hydrolysis by some constituent of the medium. In order to determine whether this constituent was present in the added serum or in the nutrient agar, the following media were prepared—

- I Infusion agar
- II " " + 5 per cent horse serum (unheated)
- III Peptone agar (no meat infusion)
- IV " " " + 5 per cent horse serum (unheated)

To these were added Andrade's indicator and 1 per cent of glucose, maltose or sucrose and slopes were inoculated with *Bact. dysenteriae* (Shiga), as this organism ferments glucose and not maltose and grows readily on simple media. Acid was produced from glucose and maltose in media II and IV and from glucose only in media I and III, sucrose was unchanged in all four media. It was evident from this result that unheated horse serum is able to hydrolyse maltose, presumably because it contains an enzyme, and further experiments with gonococci were made in order to



determine which of the other sera in common laboratory use contain this enzyme and the effect upon it of heat

#### THE ACTION OF DIFFERENT ANIMAL SERA UPON MALTOSE

##### *Technique*

The basal nutrient agar used throughout these experiments was prepared according to the method of Wright (1933, 1934) Andrade's indicator was added in bulk to the agar which was then tubed in 5 c c quantities and sterilised in the autoclave at 10 lb pressure for ten minutes Maltose was prepared as a 5 per cent solution in distilled water, sterilised by Seitz filtration and added to the melted agar—cooled to 40-50° C.—in the proportion of 1 c c to 5 c c of agar The maltose solution, if not used immediately after preparation, was stored in the ice chest

The sera were sterilised, when necessary, by Seitz filtration and stored in the ice chest With the exception of the horse serum, which was supplied by Messrs Burroughs Wellcome and Co Ltd, all were obtained from clotted blood For use 0.25 c c of serum was added to 5 c c of maltose agar in  $6 \times \frac{5}{8}$  inch tubes The medium was then well mixed and sloped and was used the same day or after storage overnight in the ice chest In all experiments control media were included containing 1 c c of distilled water in place of the maltose to check the absence of fermentable carbohydrate in the serum All experiments were done in duplicate

Considerable difficulty was experienced in obtaining good growth with freshly isolated gonococcus strains on serum agar, particularly with horse serum This was finally obviated by using a thick suspension in broth of a 24-hour blood agar culture Of this a loopful was planted on the serum medium and the tubes were closed with rubber caps which were left in position for 24 or 48 hours and then removed In most cases a satisfactory growth was thus obtained It is not yet known whether the virtue of the rubber cap lies in the retention of moisture or of small amounts of CO<sub>2</sub>, or both That CO<sub>2</sub> is produced in the closed tubes is evidenced by the development of a pink colour in the medium which disappears on further incubation, after the removal of the caps, from all tubes in which no fermentation has taken place

All experiments were carried out at 37° C After the 1st or 2nd day, the caps were removed from the tubes and incubation continued until the 5th day, when the final readings were taken All the gonococci tested (nine strains) were obtained from cases of acute gonorrhoea It is quite certain that these strains were non-maltose fermenters and that the fermentation observed in certain maltose-containing media was due to a preliminary hydrolysis of the maltose

##### *Hydrolysis of maltose by unheated sera*

*Sera from different species* Unheated sera from five different species were tested, namely human, rabbit, sheep, horse and ox, and four strains gave a satisfactory growth on all five serum media Acid was produced from maltose in the presence of sheep, horse and ox serum but no fermentation occurred with human and rabbit serum

*Sera from different individuals* Unheated sera from six different horses (all normal) and six different rabbits (three normal and three injected with leptospiræ) were tested to determine whether the action upon maltose was constant for these two species Two strains were tested in each case Acid was produced from maltose in the presence of the horse sera whereas

no fermentation occurred in the presence of the rabbit sera. In addition, as many workers use ascitic fluid in carbohydrate media for the gonococcus, samples of ascitic fluid were obtained from three different patients and incorporated in 5 per cent and 30 per cent concentration in maltose agar. Two strains were tested on these media and no acid production occurred.

*The effect of heat on the activity of serum maltase*

Unheated sheep, horse and ox sera behave as if they contain a maltase, it was considered desirable to test the effect of heat on this activity. To avoid technical difficulties arising from coagulation of the serum at high temperatures the heated serum media were prepared by adding 5 per cent unheated serum to nutrient agar and subsequently heating in a water bath to the required temperature. The maltose solution was added to the serum agar after heating to avoid decomposition.

*Heating to 55° C for half an hour.* Three strains were tested on sheep, horse and ox serum media (a) unheated, and (b) heated to 55° C for half an hour. Acid was produced from maltose in both sets of media.

*Heating to 100° C for five minutes.* Three strains were tested on sheep, horse and ox serum media (a) unheated, and (b) heated to 100° C for five minutes. Acid was produced from maltose in the unheated serum media but no fermentation occurred in the heated.

*Heating to 55°, 65° and 75° C for ½, 1, 2 and 4 hours.* Horse serum was the only serum tested. It was found that four hours' heating at 55° C had little or no effect on the activity of the horse serum but two hours' heating at 65° C and half an hour at 75° C appeared to inactivate the serum maltase. With regard to the heated sera it must be noted that, although the inactivation of the enzyme brought about by certain degrees of heating is considerable it is probably not complete. The results given were obtained with the gonococcus as test organism but two experiments made with *Bact. dysenteriae* (Shiga) on sheep, horse and ox serum heated to 100° C for five minutes showed slight acid production in some of the tubes. Shiga's bacillus is a more active fermenter than most strains of gonococci and probably detects traces of hydrolysed maltose which are insufficient in amount to give measurable acid production with the gonococcus.

### DISCUSSION

The presence of a maltose splitting enzyme in certain animal sera was known to Hiss (1901 05, p. 330) who says, when discussing the preparation of beef serum water, "Glycogen is readily acted upon by the diastase of the blood, and subsequently by the maltase, if the preparation be not rapidly carried on or the serum water heated previous to its addition." The only direct reference so far found to difficulties caused by this activity in work with gonococci is that of Rosier (1936) who states that "many strains" of gonococcus produce acid from maltose in the presence of horse serum.

In spite of the irregular results obtained with maltose in some of the early investigations (Wollstein, 1907, Gurd, 1908, Arkwright, 1909, Watabiki, 1909) only one of these workers used a medium which undoubtedly contained an active serum maltase. This was Watabiki, who tested 15 strains of gonococci in carbohydrate peptone water containing 50 per cent horse serum heated to 55-60° C for 40 minutes and found that they all fermented maltose. The fact that this maltase activity of certain sera seems to have been overlooked in this connection until recently (Rosier) can only be attributed to the preference of most workers with gonococci

for fermentation media containing enrichment fluids from which the enzyme is absent or in which it has been fortuitously inactivated. Thus human serum was used by Martin (1910-11) and Tulloch (1922) and ascitic fluid by Dunn and Gordon (1905), Elser and Huntoon (1909), Torrey and Buckell (1922), Anderson, Schultz and Stein (1923), Lentz and Schafer (1936) and Cohn (1936). Cole and Lloyd's fermentation medium (1916-17) was prepared with sheep blood but this was coagulated by steaming for one hour, a process which would certainly inactivate the maltase originally present. Similarly Cohn used a modified Levinthal agar containing, in addition to ascitic fluid, defibrinated beef blood coagulated by steaming for ten minutes and his strains produced no acid from maltose. Lentz and Schafer, while obtaining regular results with ascitic fluid medium, found that in the presence of white of egg the gonococcus fermented maltose and laevulose as well as glucose.

In this investigation the practical value of rabbit serum agar as a basal substrate for gonococcus fermentation tests has become very apparent. In addition to lacking the maltose-splitting enzyme, rabbit serum favours the growth of the gonococcus and seems to be preferable in this respect to human serum. It is not, however, warranted to draw conclusions as to the relative nutrient value of different sera from the results obtained in this investigation, as the experiments were all made with mass inocula. Suffice it to say that 5 per cent rabbit serum agar containing 1 per cent of the carbohydrate and used in capped tubes as described above has given very satisfactory results for routine fermentation tests on the freshly isolated strains of gonococci so far examined.

#### SUMMARY

1 Sheep, horse and ox sera contain a maltose-splitting enzyme which is apparently absent from rabbit and human sera and from ascitic fluid.

2 Exposure to 55° C for half-an-hour (sheep, horse and ox serum) does not decrease the activity of the enzyme, and horse serum heated to 55° C for four hours retains its activity. Inactivation is produced at 100° C in five minutes (sheep, horse and ox serum), at 75° C in half-an-hour (horse serum) or at 65° C in two hours (horse serum).

3 A medium containing 5 per cent unheated rabbit serum is a suitable basis for routine fermentation tests with gonococci.

4 It is advantageous to incubate cultures in tubes sealed with rubber caps for 24-48 hours after inoculation.

I am indebted to Dr D. T. Robinson for assistance in connection with certain points in this investigation.

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## OBITUARY NOTICE OF DECEASED MEMBER.

George Henry Falkner Nuttall.

1862-1937

(PLATE XXII)

NUTTALL was descended from an old Lancashire family which settled in Ireland in 1707. His father, Robert Kennedy Nuttall, M.D. (Aberdeen), went to Australia but in 1850 migrated to San Francisco and practised there till 1865. He married Magdalena, daughter of John Parrott of San Francisco, and their son George was born there on 5th July 1862. In 1865 the family came to Europe and remained there till 1876, the children being educated in England, France, Germany and Switzerland. Nuttall therefore spoke several languages and was subsequently perfectly at home in foreign countries. His knowledge of languages also helped him greatly in his future work.

Most of the family returned to San Francisco in 1876, where his father died in 1881. Nuttall returned in 1878 and obtained the degree of M.D. of the University of California in 1884. He was present in San Francisco at the time of the great earthquake and was enrolled amongst the armed special constables, whose main duty it was to prevent and stop looting. He often gave graphic accounts of his experiences at that time.

In 1884-85 he travelled with some members of his family for a year in Mexico, sometimes by leave of the Government, sometimes by leave of the bandits. The state of the country is illustrated by one of his experiences. A little distance outside a large town he saw a group of small wooden crosses and asked what they were. "The graves of some of the travellers, murdered by bandits" was the answer. A little further on was another group of crosses. On enquiry, the answer was "Those are the graves of the bandits". His sister, Zeila (1857-1933), devoted her life to Mexican archaeology.

In 1885-86 Nuttall went to the newly founded Johns Hopkins University, Baltimore, and worked at biology under H. Newell Martin, who had been one of Huxley's demonstrators.

In 1886 he went to Germany and, after spending a few months at Breslau, removed to Göttingen where he remained for four years, working under Flügge. Most of his time was devoted to the study of botany and zoology, which exerted a predominating influence on

his career, for the greater part of his life was devoted to parasitology. At that time however, there was in progress a hotly debated controversy on the causes of immunity and Nuttall took up this question. Though it had been shown by Fodor and others that bacteria injected into the blood stream disappeared Nuttall (1888), working in Flugge's Institute, was the first to demonstrate that defibrinated blood possessed a considerable bactericidal power against anthrax bacilli, and that this power was destroyed by heating to 55° C and also disappeared in time. For an essay based on this work "A contribution to the study of immunity," Nuttall received the triennial Boylston prize from Harvard University. This was the first of Nuttall's several pioneer researches, which in his own and other hands led to such fruitful results. The essential facts revealed by Nuttall were confirmed and extended by others, and within two years antitoxic immunity had been demonstrated by Behring and Kitasato (1890). He graduated Ph.D. at Gottingen in 1890.

After travelling for a year Nuttall returned to Baltimore in 1891 as assistant to W. H. Welch, the professor of pathology, and in 1892 was made associate in hygiene. In 1891 he published the first accurate method for estimating the actual number of tubercle bacilli in the sputum, as well as a method for inoculating media and animals with known numbers of bacteria. In 1892 he published in conjunction with Welch a very full account of *B. aerogenes capsulatus* now known as *B. welchii*, an organism the importance of which as a pathogenic agent was not fully appreciated until the Great War.

In 1892 he visited Havana and was greatly impressed with the heavy mortality (25 per cent.) from yellow fever amongst the troops sent out from Spain and the relative immunity of the native population. The cause and mode of transmission of the disease were then quite unknown.

In March 1893 he again went to Europe, this time to prepare himself for the professorship of hygiene at Baltimore, which had been offered to him, but, after meeting his future wife in Dresden, he altered his plans and remained in Germany till 1899. He first worked at Gottingen for about a year and then, as a voluntary assistant, at the Hygienic Institute, Berlin, under Max Rubner. In 1895 he married Paula, daughter of Kammerherr von Oertzen-Kittendorf of Mecklenburg.

During this period he carried out with Thierfelder (1895-97) an important research into the possibility of animal life in the absence of bacteria. After overcoming many technical difficulties they demonstrated that it was possible to bring up guinea-pigs, delivered by Caesarean section and kept in a specially devised chamber, free from contamination by bacteria. Owing to the

great labour involved the animals were kept alive for two weeks only, but were then quite healthy. In the course of these experiments it was shown that some of the substances found in the urine arise from bacterial activity in the intestinal tract.

In 1895 he designed his well known thermostat to contain a microscope for the study of bacteria, etc. at known temperatures.

In 1897 he first turned his attention to the part played by insects, ticks, etc., in the spread of disease, and in 1899 published his well known critical and historical monograph, "On the rôle of insects, arachnids and myriapods as carriers in the spread of bacterial and parasitic diseases of man and animals." At that time the insect transmission of plague and yellow fever was not suspected, of malaria it was strongly suspected but not convincingly demonstrated, but the relation of mosquitoes to filariasis and of ticks to Texas fever in cattle was known. Very few of the books on hygiene even mentioned the possible association of insects with disease, and Nuttall's monograph stimulated research, though the widespread importance of the subject was not fully appreciated for some years.

In 1899 Nuttall was invited to Cambridge by Sir Clifford Allbutt, regius professor of physics, who suggested that a teaching post might be created for him, though he warned him that the stipend of a university lecturer was only £50 a year. Nuttall, who had been greatly attracted to Cambridge on previous visits, came during the long vacation of 1899, and gave a course of lectures on bacteriology. In 1900 he was appointed university lecturer in bacteriology and preventive medicine for a period of five years and received an honorary M.A. degree. At that time the staff of the Department of Pathology consisted of the professor, G. Sims Woodhead, and the demonstrator, Strangeways Pigg, who soon afterwards changed his name to T. S. P. Strangeways. The department was housed in an old, small and dilapidated building in which two of the rooms were subdivided by match boarding into small research rooms or compartments. Nuttall occupied one of these compartments, about 12 x 7 ft., on the ground floor. In this small but well lighted room he worked till 1904 and carried out some of his best known researches, namely those on precipitins and malaria.

He did not discover the precipitins, but he very soon realised their importance and confirmed and extended the observations which had been made (1901). Then for three years he worked with great diligence on the application of the precipitin test to the relationships amongst vertebrates, and in 1904 brought out his classical monograph on "Blood immunity and blood relationship," in which he clearly demonstrated group reactions amongst the Mammalia and a bond between birds and reptiles. Under his



supervision the first experiments on the application of the test to forensic medicine were also carried out

In 1900 he became interested in the history of malaria in Great Britain, and with others undertook a survey of the distribution of *Anopheles* in England. With the aid of maps, used for the first time for showing the distribution of disease-bearing insects, he demonstrated that three species were widely distributed, but were most common in low-lying lands where malaria had been common. The disappearance of the disease was therefore not due to the extinction of *Anopheles*. Then with Shipley he made important researches into the structure and biology of *Anopheles*, constituting the most detailed study of its kind which had been published and revealing many new facts.

From 1900 to 1906 he lectured on bacteriology to DPH candidates and students preparing for the final MB examination. His teaching was extraordinarily stimulating, for he not only spoke with a great personal knowledge of his subject, but knew all the leading bacteriologists of the time and recounted anecdotes illustrating their characters, habits and researches. At tea-time he joined in all subjects of conversation and often told tales of his hunting trips and other experiences.

In 1901 he founded the *Journal of Hygiene* and edited it till his death. He was very proud of the Journal and spared no trouble in editing. As an editor "Not only did he require honesty, he required intelligibility and did much to raise the standard of literary workmanship in medical and biological circles."

When the new medical school buildings were opened in 1904 Nuttall occupied a moderate sized room on the top floor, where he worked till 1907. In 1906, in consideration of "his services to the University and his distinction as a man of science" he was appointed reader in hygiene at a stipend of £250, but only held the appointment for a few days, for on 16th October he was elected the first Quick professor of biology. The regulations relating to this research professorship unfortunately prohibited further teaching of students, and the very small grants towards maintenance and assistance hampered him throughout his 25 years' tenure of this post, and imposed the necessity for continual begging for funds outside the University.

In 1907 the Quick department moved into a large room (63 × 37 ft) under the Humphrey Museum. This room, which he divided into alcoves by suitably placed cupboards and screens, was dusty, noisy, uncomfortable and in many other ways unsuitable, but here Nuttall worked with great energy and enthusiasm till 1921, mainly on "red-water" in animals. With the aid of grants and benefactions, as well as contributions from his own stipend, he established four paid posts, and all available places in the

laboratory were generally occupied by research workers. In 1908 he founded *Parasitology* and edited this journal till 1933, when Keilin took over the editorship.

In 1904 he infected dogs with *Piroplasma canis* by means of adult infected ticks (*Hæmophysalis leachi*) sent from South Africa by Lounsbury—the first case of investigation of a disease imported by means of an infected vector. In the following years he worked out its mode of multiplication in the living blood, compared this with that of other species, and attempted to ascertain its life history in the tick. Experiments on drug treatment led to the discovery that trypan blue, which has since been used throughout the world, was both a preventive and a cure for the deadly piroplasmoses of dogs, cattle, sheep and horses. He also worked on the spirochaetoses and on East Coast fever in horses.

In 1908 he published with L. E. Robinson an important paper on the structure of the tick, *Hæmophysalis punctata*, and shortly afterwards the first fasciculus of his well known monograph on "Ticks" appeared. Work on the classification, biology and disease-transmitting capacities of ticks occupied most of the rest of his professorial career, and he amassed a very fine collection containing many types.

In 1909 accommodation for experiments on large animals became necessary and in conjunction with the professors of pathology and agriculturo, Nuttall bought about 25 acres of land in the Milton Road and erected research buildings. The land and buildings were conveyed to the University in 1913, and in 1923 were allocated to the newly established department of animal pathology.

During the Great War he turned his attention to the louse problem, and did much work on the anatomy, biology and relation to disease of human lice and on preventive measures against them. He published numerous papers on this subject and wrote the *Army Council Instruction* (1918) on "Lice, their relation to disease, biology and means of combating lousiness among soldiers."

Following an appeal for funds by Nuttall in May 1919 Mr and Mrs Percy Molteno very generously intimated in October their willingness to present to the University a sum of £30,000 for the purpose of erecting an institute for parasitological research in Cambridge. This benefaction was gratefully accepted, and in 1921 the Molteno Institute was opened and the Quack department moved into it. Here Nuttall continued his work, but the supervision of the Institute, the direction of research and the necessity for raising funds to meet the increasing expenses limited his personal researches and he published few papers, though he collected much valuable material for further fasciculi, which he had planned, on ticks.

In 1931 he resigned the Quick professorship and became emeritus professor of biology. During his tenure of the professorship he had raised £60,000 and received grants totalling £12,000. In all, 314 papers had emanated from his laboratory.

Nuttall during his career published more than 200 books and papers dealing with several branches of biology, including hygiene, bacteriology, immunology, protozoology and parasitology, and formed a large and valuable library, most of which he presented to the Institute. Nuttall received many honorary degrees from foreign universities and the Belgian Order of Leopold II and the French Order of Commandeur de la Légion d'Honneur. He was elected F.R.S. in 1904. He was a member of several important Government committees and represented the University at many congresses. In 1932 he was presented by his colleagues and pupils with his portrait in oils by de Lazló.

Nuttall was a most enthusiastic, conscientious and industrious worker, and the gift of recognising the potentialities of observations of apparently little importance made him a pioneer in several lines of work. After his election to the Quick professorship he ceaselessly advocated the importance of parasitology, but he encountered much prejudice and indifference and had a hard struggle to establish the Molteno Institute. He had, however, the pleasure of seeing parasitology recognised and properly taught as an important part of the public health and tropical medicine diplomas, and of knowing the high repute in which his department was held both at home and abroad.

Nuttall was alert in his movements, eager and vivacious in his talk, and kind, courteous and considerate to all. He had a charming manner and winning smile and was most loyal to his friends. He was singularly modest and regarded the many distinctions conferred upon him as recognition of his subject rather than of himself, and his knowledge of several languages enabled him to make innumerable friends in many countries. He was an excellent draughtsman with a great interest in art, and had a considerable knowledge of heraldry and gardening.

An original member of the Society, Nuttall was elected an honorary member in 1933, a distinction of which he was highly appreciative.

He died suddenly (16th December) on the eve of a dinner to be given in his honour on his retirement from his long and successful editorship of the *Journal of Hygiene*.

His wife died in 1922, he is survived by two sons and a daughter. The photograph reproduced on plate XXII was taken in 1936.

G. S. G.-S.

## BOOKS RECEIVED

### Physiological chemistry of the bile

By HARRY SEBOTKA London Baillière, Tindall & Cox 1937  
Pp xii and 202, 4 text figs 13s 6d

Most readers of this monograph will feel that, in spite of its undoubted value, it suffers from a grave omission. It is a work on the bile without any treatment of the subject of the bile pigments. In the preface the author explains this omission by saying that the chemistry and physiology of the biliary pigments was excluded, as it (*sic*) could not be treated adequately without an extensive exposition of the physiology of the blood pigments as well as the chemistry of pyrrol derivatives. Yet the "biochemistry of cholesterol has been included to that extent which was justified by virtue of its occurrence in the biliary secretion." Surely this reasoning applies with at least equal force to the bile pigments.

In spite of this omission however, the book will be welcomed by all who have any special interest in the bile. It contains a wealth of information and a bibliography of some twelve hundred references. Nor is it a mere catalogue: the subject matter is both well arranged and readable. A number of minor errors and omissions suggest hurried revision and there is excessive use of alternative terms, but these faults do not detract from the essential value of the book, nor conceal the industry which has gone to its making.

### Practical methods in biochemistry

By FREDERICK C KOCU Second edition London Baillière, Tindall & Cox 1937 Pp ix and 302, 18 text figs 10s

This book, intended for medical students, gives a course of practical work in biochemistry in a clear and well arranged manner. In some respects it goes beyond the requirements of the ordinary medical undergraduate, for whom the course might with advantage undergo a certain amount of pruning by his teacher. This is no detriment to the book as a whole, since it increases its value to readers other than those referred to, the clinical biochemist for example will find its wide selection of tests and analytical methods of distinct usefulness.

The work is practical throughout, and makes no attempt to interpret the significance of the results of blood and urine analysis from the clinical or pathological standpoint. Its claim to comprehensiveness is marred by certain omissions. Thus there is no reference to the chemistry of the cerebrospinal fluid, the faeces or concretions, and no methods are given for the determination of phosphatase or diastase.

**The avitaminoses The chemical, clinical and pathological aspects of the vitamin deficiency diseases**

By WALTER H EDDY and GILBERT DALLDORF London Baillière, Tindall & Cox 1937 Pp ix and 338, 49 figs on 29 plates and 3 text figs 20s.

This successor to the senior author's *The vitamine manual*, written some fifteen years ago, has a somewhat different scope from that of most of the books lately published on the subject of the vitamins. In the present volume the pathological changes and clinical symptoms resulting from mild or severe grades of deficiency form the main theme, the chemical aspects of the vitamins being kept rather in the background and occupying not more than a quarter of the text. The tissue changes associated with deficiency of each of the better known vitamins are described in considerable detail and are illustrated by excellent photographs. Special emphasis is laid on the lesions which occur in the early stages of the deficiencies. The clinical sections fulfil the double purpose of collecting available accounts of well recognised deficiency syndromes and of drawing attention to less well established states of dietary deficiency with which the vitamins may or may not be intimately concerned. The general plan of arrangement is as follows. An account of the chemical nature and functions of each vitamin is followed by a description of the clinical symptoms caused by gross deficiency of the vitamin. The histological changes associated with such deficiency are then described and, finally, the possible relation of milder grades of the deficiency to morbid states not universally regarded as connected with dietary faults is discussed. Short sections at the end of the book are devoted to methods of assaying vitamins and to clinical tests for estimating the degree of saturation of the body with vitamins A and C. A table is also included giving the amounts of the vitamins present in common foods.

In a book dealing with a subject which has grown in a short space of time from nothing to one of the most popular fields of medical investigation, it is bound to be difficult to pick out the most significant observations and to apportion fairly the credit for important advances. On the whole these difficulties have been overcome with considerable skill. If the reader is sometimes apt to wonder whether too much or too little stress has been laid on some particular piece of work, he will find a short bibliography at the end of each chapter indicating some of the sources of information on which the writers' views are based. It is a pity that obvious but annoying slips such as the repeated spelling of phosphorus as "phosphorous" have been allowed to go uncorrected, for on the whole this book will be found to belong to the class of readable books.

**An introduction to bacteriological chemistry.**

By C. G. ANDERSON. Edinburgh: E. & S. Livingstone 1938. Pp viii and 276. 2 text figs. 10s 6d

In his preface Dr Anderson disclaims any attempt to provide an encyclopaedia and states that his object has been to "cover the requirements of students, and perhaps of those research workers whose interests may not be primarily chemical but who feel the need for some understanding of the metabolic behaviour and chemical nature of the organisms which they are handling." The aim is one with which many

bacteriologists will have the greatest sympathy and particularly those whose duties include the teaching of bacteriology to non medical students or whose researches lead them, perhaps inadequately equipped with chemical knowledge and technique, into some of the most difficult fields of chemical study. The last few years have seen a most healthy and profitable incursion by the chemist into the field of microbiology, an invasion which has been profitable to both chemistry and microbiology and whose end is not yet. Anything therefore which tends to interest the chemist in our bacteriological problems is to be applauded and this volume should certainly do that for the student of chemistry. It is an introduction to the subject, presuming a certain amount of knowledge of organic chemistry and of bacteriology and covering a very wide field in remarkably small compass.

Part I (56 pp) deals with general consideration such as pH, oxidation reduction potentials, colloids, enzymes and the chemical composition of bacteria, yeasts and fungi. Part II (161 pp) is concerned broadly with bacterial metabolism, the growth requirements of various types, certain physiological activities and the results thereof, industrial applications and finally the peculiar constituents of various kinds found in bacteria. Then follow two short chapters on immune chemistry and a very brief one on chemical methods.

Much of the information contained in this volume can only be got with great difficulty from original sources, and many workers will be grateful for its compilation in this handy form. For those who want more, references are given to more elaborate monographs. One feels that these lists might well have been extended and the student guided to the originals. Indeed the references are perhaps the least satisfactory part of the book and it is to be hoped they will be supplemented in a second edition. The last chapter too is in a very larval form and if not extended by detailed descriptions of methods might well be supplemented by references to accounts of tried and approved methods.

One notes a few misprints, "C xerose" (p 71), "et alia" (p 178) and "polysaccharides" (p 220), and there is a certain picturesqueness about the statement that nitrogen fixation has been known "from time immemorial" (p 111). It seems hardly accurate to say that ammonium phosphate is a good source of nitrogen for all microorganisms except *Vibrio cholerae* (p 108) or that the pneumococcus, meningococcus and gonococcus belong to the *Haemophilus* group (p 71). The statements on pp 220 and 231 about the action of formalin on antigens are sufficiently contradictory to confuse the student and to require elaboration. These and one or two grammatical errors (p 70, line 28) are minor blemishes in a work which, within the limits set himself by the author, is both welcome and competent.

#### The microscope theory and practice

By CONRAD BECK London R. & J. Beck, Ltd 1938 Pp 264, 217 text figs 7s 6d

It is not often that a book on the microscope is written by one who has intimate knowledge of its mechanical and optical construction and yet is sufficiently interested in its use to master the necessary manipulative technique. Mr Conrad Beck has these combined qualifications, to which his book bears testimony on every page. Actually it is a new edition of his two previous books on the same

subject, one of which was elementary, the other advanced. The volume now noticed is not more in bulk than either of its predecessors, but drastic revision has enabled the author to convey more information without loss of value, indeed, it has resulted in an increase of lucid information for the general reader. The chapter on geometric optics is of interest, but is not essential for an adequate working knowledge of the use of the microscope in any branch of medicine. Chapter III on aperture and resolution is perhaps the most useful section, as it conveys the information without which no microscope user is likely to get the best results. Interpretation is the essence of all observations with the microscope, and this depends on accurate adjustment of the optical components with which the observations are made. Chapter IV is hardly less important, it includes a description of work upon a subject that the author has made particularly his own. Much of this chapter is devoted to original observations. It is not always easy reading for those unfamiliar with optical principles, but no one need be afraid of it, much will be gained from even a partial appreciation of the experimental results described. Chapter V is devoted to illumination and technique, matters which may be regarded as the basis of accurate observation. Unless regard is paid to illumination and to the accurate centration of each part of the illuminating system, it is not possible to secure that close resemblance of image to object on which all observations with the microscope are dependent. Later chapters are devoted to a description of typical microscopes, mainly those produced by Messrs R & J Beck, Ltd, together with interesting accounts of the apparatus used for ultraviolet microscopy, and on the use of polarised light. The book may be commended to those wishing to secure a sound working knowledge of the principles and practice of microscopy.

J E B

#### A monograph on veins

By KENNETH J FRANKLIN. London. Baillière, Tindall & Cox. 1937. Pp xxii and 410, 45 text figs and a frontispiece. 27s.

In this monograph Dr Franklin sets out to summarise what is known of the anatomy, embryology (by Mr Keith Richardson), physiology, pharmacology and pathology of veins. He has been stimulated to do this because, as he says, "the sole large part of the circulatory system, which has not yet received its proper share of attention is the venous system." When the reader, after traversing 350 pages of text, is confronted with 54 pages of references, he may wonder whether this statement is strictly accurate, nevertheless it is probably true to say that there has been a tendency in the past to under-estimate the importance of the veins in maintaining the circulation.

Monographs are chiefly of value when, by developing a thesis, they stimulate thought and research on a chosen subject; and when, by accumulating and arranging scattered but inter-related work, they act as works of reference. To some extent Dr Franklin's work fails in both of these objects. The chief physiological importance of the veins is probably their capacity to accommodate large and variable amounts of blood and thus to influence the inflow to, and output of, the heart; something of their function in this respect is known but much remains to be discovered, particularly in adding precision to our ideas. One might think that this would make a simple *motif* around which the book could be constructed, but though Dr Franklin reviews the question of

blood reservoirs in chapter VI, the reader has to wait for 90 pages until in chapter XIII the heart and venous return are dealt with in the space of 5 pages. As a work of reference Dr Franklin's monograph is more successful but here again it would have been of much greater value had it been better arranged and the writing more concise and precise. One example of faulty arrangement has been cited. A second may be chosen from chapter VII where on pp 99-103 the author considers the function of the *rete mirabile* found in certain diving mammals; not till the final paragraph does he indicate what the *rete mirabile* is, and even then the description is so imperfect that the reviewer was forced to consult an independent work before the argument became at all intelligible. A third may be chosen from the three chapters entitled "Veins and the nervous system," where we find considered subjects which are unrelated to the nervous system, such as the reactions of the veins to mechanical stimuli and changes of temperature, and of the veins, liver and spleen to various chemical agents. Another criticism of the work is that the author attempts to deal with aspects of the subject with which he is not familiar. Thus chapter XXII, entitled "Cluical," is an account of the pathology of veins which in many respects is so trivial that it would have been better omitted. These are the chief faults of the book and they are faults which can be eliminated with care. But the monograph has its virtues too. Thus it is attractively bound and printed and the illustrations are chosen and reproduced well. In its preparation it is evident that Dr Franklin has read widely and those who are interested in his subject will be grateful to him for gathering together for the first time in a volume in the English language a comprehensive summary of what has been written on veins.

#### American medicine, expert testimony out of court

Edited by ESTHER EVERTT LAPP. New York. The American Foundation. 1937. Pp lxxix and 1435 (two volumes). \$3.50.

This is a very detailed enquiry undertaken by the American Foundation as to whether the population as a whole is receiving "adequate medical care." And if not, why not? It is a laborious publication in two bulky volumes compiled from the information supplied by questionnaire. Two sections of the medical profession have been consulted: (1) those in practice for over twenty years, and (2) those who have graduated for not more than five years. The contributors are named but their names are not attached to the various excerpts from the mass of replies.

All aspects of medical practice are dealt with: general and specialist practice, team work, hospitals, state service, public health, etc. The defects in medical education are also exhaustively reviewed and many suggestions for modification and improvement are made. There is no doubt that it is a mine of informing opinions about the matters dealt with and will doubtless be stimulating and useful to all would-be reformers in matters connected with the practice of medicine, whether they be doctors, statesmen or even—may it be said?—cranks. Whether such usefulness will be commensurate with the labour of compilation must at best be doubtful: there is the further uncertainty as to whether the sources of the material are the most reliable and practical that could be obtained. To most readers I think the volumes will appear as the dictionary did to the man discovered avidly reading it: "I think the stories are very interesting but they are rather short."

W. H. V. T.



**Recent advances in pathology**

By GEOFFREY HADFIELD and LAWRENCE P. GARROD Third edition  
 London J & A Churchill 1938 Pp xii and 420, 153 figs on  
 48 plates, 18 text figs 15s

The third edition of this invaluable *vade mecum* appears after an interval of four years. Changes have been extensive, and little more than half of the previous text remains. Two chapters and five sections have disappeared and new chapters have been added on resistance to infection (including a discussion of the nature and significance of bacterial allergy) and on reticulosis and the reticulo-sarcomata. The chapters on deficiency diseases and on cancer have been subjected to extensive alteration, and those on experimental cancer research have been largely rewritten. A bold attempt has been made to review, in four pages, the present position of the cancer problem as a whole. The authors are critical of the virus hypothesis as applied to mammalian tumours. "it remains broadly true that, although a certain very restricted class of tumour can be produced by an agent separable from its cells, the whole range of mammalian malignant growths has steadily refused to betray its possession of such an agent. No amount of ingenious argument can explain this away there must be some radical difference between the filtrable fowl tumours and growths in mammals." They suggest, as a working alternative hypothesis, that "malignant disease is not aetiologicaly a single entity at all, but a type of reaction to a variety of stimuli, usually chemical and possibly sometimes microbic. That this reaction is irreversible may well be due to the altered properties of the cell itself, and does not necessarily prove the continued presence in it of an extraneous agent."

The chapter on the respiratory system includes an excellent review of the present state of knowledge of silicosis and allied diseases, though the typical "asbestosis body" is wrongly described as consisting of "a bulbous extremity, a tapering body composed of a series of discs with rounded margins, and a slender tail" (reviewer's italics). Tails when present are due to fracture.

Extensive additions have also been made in connection with the subjects of gastritis, the relationship between gastro-intestinal functions and anaemia and disorders of the adrenal and pituitary glands.

The authors' chief aim has been "to deal fully with a restricted range of those subjects whose study has made important progress." At the same time they have felt it necessary to retain certain chapters on subjects in which there has been comparatively little change in order to avoid losing balance and to prevent the volume from becoming a series of isolated essays. The authors are certainly to be congratulated on a very successful achievement. The book will continue to be of the greatest value to teachers and students of pathology alike, and to all those members of our profession who desire, without undue labour, to keep abreast of present-day pathological research.

**Handbook of practical bacteriology**

By T. J. MACKIE and J. E. MCCARTNEY Fifth edition Edinburgh  
 E. and S. Livingstone 1938 Pp xi and 586, a few text figs 12s 6d

Three-and-a-half years ago we offered a warm welcome to the fourth edition of Mackie and McCartney (this *Journal*, 1934, xxxix 543), and we find the new edition equally deserving of our commendation. It now attains the status of a "Handbook" instead of an "Introduction" and it has increased its stature by eighty-two pages.

# The Journal of Pathology and Bacteriology

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## THE PATHOGENESIS OF PULMONARY SCHISTOSOMIASIS IN EGYPT WITH SPECIAL REFERENCE TO AYERZA'S DISEASE

A F BERNARD SHAW and A ABOU GHAREEB

*From the Department of Pathology of the Egyptian University, Cairo*

(PLATES XXIII-XXXV)

It has been estimated that 60-70 per cent of the 14½ million inhabitants of Egypt, of whom about 80 per cent are agricultural workers or fellahoon, are infected with schistosomiasis. Since Bilharz discovered the cause in 1851, this disease has been the subject of intensive study in all its aspects and not least as regards the lesions in the various organs. It has been known for many years that bilharzial ova may be found in the lungs but the frequency and importance of this complication have hardly yet received recognition. It will be shown in this paper that in at least 33 per cent of cases of bilharzial infection there is pulmonary involvement, and that in 2 per cent these lesions are the immediate cause of death. Of the three schistosome worms parasitic in man only *S haematobium* and *S mansoni* occur in Egypt.

### *Material*

This consists of 282 autopsies on cases of schistosomiasis admitted to the University hospital (Kasr el Aini). About 80 per cent of these patients died of other causes than bilharziasis.

### *Methods*

At autopsy practically all the organs were inspected and examined microscopically but only the changes which bear upon the pulmonary lesions are included here. In interpreting the lesions in the lungs the routine method of opening these organs is important. The bronchus and its two divisions

are cut open with scissors, the knife laid in the floor and the organ divided from hilus to periphery. The bronchi and vessels are thus exposed parallel to their long axes and the histological sections conform with this. A block for microscopical examination was taken from each lung and in special cases multiple blocks parallel with and transverse to the vessels were secured. Of the lungs alone nearly 900 sections were studied. To identify the species of ova in the lungs, pieces were macerated in 3 per cent KOH. For other organs material was scraped from the cut surface. In our experience the species of ova can rarely be determined in sections. The ovum measures  $150 \times 60 \mu$ , and as identification depends on the position of the spine it is obvious that in sections of the ordinary thickness the chances are all against the spine of a particular ovum being visible in a given section. Moreover shrinking often produces appearances in the shell simulating a spine.

In studying the reactions of the tissues to the ova it would be valuable to distinguish a living ovum from one which has recently died. At present this is impossible, as the only criteria of death are calcification or destruction of the contents of the shell by giant cells, events which occur long after the death of the embryo.

Photomicrographs of bilharzial lesions are often unsatisfactory, partly due to differential shrinking, which leaves a space round the ovum, partly to the chitinous shell, which causes the ovum to be torn from its bed by the microtome. Calcified ova are especially liable to be dislodged and carried into situations where their presence may be misinterpreted.

#### STRUCTURE, HABITS AND EFFECTS OF THE PARASITES IN RELATION TO PULMONARY INFECTION

The male worm of *S. haematobium* is about 15 mm long by 1 mm broad, the female, usually carried in the gynæcophoric canal of the male, is about  $20 \times 0.25$  mm. The worms of *S. mansoni* are rather smaller. Both species are strict intravenous parasites and are attached to the wall of the vein by two suckers. Migration along the vessel wall is also effected by the suckers. Worms which lose their hold become emboli. *S. haematobium* has the greater egg-laying capacity. In each species the elliptical ovum measures on an average  $150 \times 60 \mu$ . The ovum of *S. haematobium* has a short terminal spine, in *S. mansoni* the spine is lateral and about  $20 \mu$  long. The shell consists of chitin and when mature contains an embryo (miracidium) with two glands opening by ducts at the cephalic end. The ova are deposited intravenously within the smallest venule which the female can reach and then pass into the tissues. If they fail to escape from the body they eventually die and calcify. Ova which fail to engage in the vessel wall become emboli. *S. haematobium* inhabits the vesical, ureteric and prostatic venous plexuses and deposits its ova in these organs. *S. mansoni* lays its ova in the colon and rectum. One species may trespass on the domain of the other.\* In Egypt, double and mixed infections

\* In this department we define a double infection as one in which both species are present in the body but confined to their respective territories, a mixed infection one in which both species occur in the same organ.

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## Prevention of Sepsis in the Post-Mortem Room

### I

Strong post-mortem gloves, not too thick, should be worn, and should be thoroughly cleansed, sterilised and dried immediately after use. Great care should be taken to ensure that there are *no holes*. A convenient way is to fill them with water, twist the wrist portion round until it is closed off and then, by pressure, test for pin-holes. The same result may be achieved by air pressure after the gloves have been dried. In this case the glove should be turned about close to the face while pressure is exerted on the contained air.

### II

It is inadvisable to perform autopsies when there are *recent* cuts or scratches on the hands. In case of necessity, a protective covering of collodion may be used.

### III

The greatest danger of all, and the one chiefly responsible for serious and fatal infections, is *pricking the finger*. If during an autopsy the pathologist should prick himself however slightly whether with the knife or on a jagged bone or tooth, even if he is in doubt whether he has been pricked at all, it is his duty *to stop AT ONCE, remove his glove and make the wound bleed* by digital compression causing congestion, by centrifugal force (swinging the arm), by sucking and by putting the hand in hot water. There should not be a minute's delay in carrying out these procedures, and the autopsy should, if possible, be completed by someone else.

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50 copies	<i>post free</i> 6/6
100 "	" 10/6
250 "	" 21/-

especially in massive and repeated infections of the tissues and are suggestive of an allergic reaction. In other instances ova, apparently living, excite no cellular response. Only tubercle formation is described in the literature. The conditions which decide the various responses can only be elucidated by experimental methods and when more is known of the humoral state, but on the histological evidence we incline to the view that size of dose, exogenous and endogenous reinfection, immunity and allergy determine the type of reaction in a manner comparable to what Rich and McCordock (1929) have found in tuberculosis.

## PULMONARY LESIONS

### A. Lesions due to ova.

The frequency of pulmonary infection in Egypt has never been estimated. Ova of both species were found by Dew (1923) in 47 autopsies on Egyptians. He gives no figures but leaves the impression that the condition is rare.

In our series the lungs were involved in 95 out of 282 cases (33 per cent). Of these, 36 cases were chosen for identification of species. It will be seen (table I) that *S. hæmatobium* and

TABLE I.

*Species of ova present in the lungs in 36 cases of pulmonary schistosomiasis*

Species	No. of cases	Per cent
<i>S. hæmatobium</i>	21	58.3
<i>S. mansoni</i>	11	30.6
Mixed	4	11.1

In all, 69.4 per cent were infected with *S. hæmatobium*, 41.7 per cent with *S. mansoni*. This difference ( $27.7 \pm 9.7$ ) is significant.

*S. mansoni* can both infect the lungs, the former being significantly the more frequent, while mixed infections are uncommon. As for various reasons the material was selected, the figures cannot be considered as accurately expressing the relative frequency of infection. Similar results were obtained by Turner (1909) in South African natives with urinary bilharzia, *S. hæmatobium* ova being found in 50 per cent.

The route by which the ova reach the lungs from the portal and systemic veins is discussed below, but they enter the pulmonary arteries as emboli. We have never found them in the veins nor are they deposited *in situ* by the worms. In these respects the lungs differ from other organs. The number of ova is of primary

PULMONARY SCHISTOSOMIASIS



FIG 1—In the upper quadrant a parenchymatous tubercle with an avum and an adjacent respiratory bronchiole (left) and its arterial healed tubercle with eosinophilic granules but no cells visible is also seen  $\times 100$



2—A parenchymatous tubercle projecting into an alveolus. The muscular and ciliated epithelium of the respiratory bronchiole to which it is attached are visible above. The tubercle contains a giant cell but the avum has fallen out  $\times 100$



FIG 3—A parenchymatous tubercle with avum completely filling an alveolus. The arteriole lies above  $\times 145$

whole, and we have never seen areas of interstitial pneumonia attributable to bilharzia, even in massive infection, as described by Manson (1929)

In only one case in this series was an ovum seen free in the lumen of an artery and no ova were observed in the act of passing through the vessel wall. This is not surprising in view of the paucity of ova and the small area of tissue examined. Moreover embolic ova are only evident if in transit just before death. Day (1936-37) is of the opinion that the ova can reach the alveolar capillaries and readily escape into the air sacs. We are unable to confirm this. In most tissues the average diameter of the capillaries is 5-10  $\mu$  and there is no evidence that it differs greatly in different organs. Even assuming the pulmonary capillaries are twice this diameter it would be impossible for ova to enter them. It is true that ova occur free in the air sacs, especially in massive infection and when calcified, but as already stated they have been dislodged from the tissues in sectioning. In this connection the question arises as to whether ova appear in the sputum. Byam and Archibald (1923) state they may be found in severe infestation with *S. haematobium*, while Pijper (1934) found the same species once in a case of pulmonary tuberculosis. Silveira (1936) found ova in a few cases of *S. mansoni* infection. It is clear then that ova are unlikely to appear in the sputum and this accords with the essentially vascular origin of the lesions.

## 2 Focal arterial lesions

Six cases fall within this group. In addition to numerous parenchymatous tubercles these cases are characterised by arteriolar lesions focally distributed so that serial sections were necessary to demonstrate them in any number.

### Case 1

Male, aged 20. Died of bilharziasis and terminal bronchopneumonia.

**Autopsy.** Brown atrophy of heart (150 g) without hypertrophy of right ventricle. Intestines and peritoneum the site of severe bilharziasis due to *S. mansoni*. Liver (1250 g) shows typical bilharzial cirrhosis, ascites, spleen enlarged (500 g), pancreas cirrhotic. Bladder and prostate healthy and negative for ova.

**Lungs.** Ova of *S. mansoni* present. On section numerous firm greyish-white bilharzial tubercles distributed uniformly over the surface.

**Histology.** Many of the parenchymatous tubercles show necrosis, healed tubercles are scanty. Free embolic ova are present in some arterioles of about 50  $\mu$  diameter (fig 6) and some have become impacted, producing a localised acute necrotising arteriolitis with destruction of the media. The ovum may then be covered by endothelium while the subendothelial tissue and media are converted into a swollen hyaline mass of necrotic debris staining strongly with eosin (fig 7). A few pyknotic nuclei are present but there is no tubercle formation. The adventitia is infiltrated with mononuclear



## PULMONARY SCHISTOSOMIASIS



FIG 4—A conglomerate of parenchymatous tubercles with three ova and giant cells filling an alveolus and projecting into the lumen of a respiratory bronchiole  $\times 110$

FIG 5—Healed parenchyma of tubercle in which are broken and calcified ova  $\times 200$

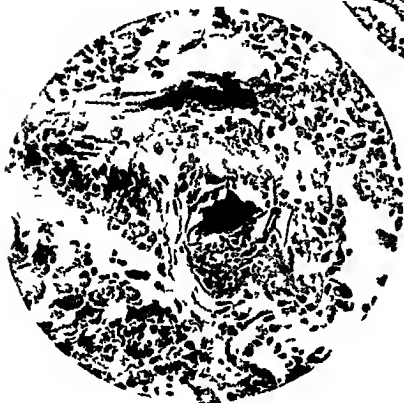


FIG 6—Case 1 Ovum of *S. mansoni* lying free in an arteriole, the lumen of which is  $50\mu$  in diameter. The muscular coat of the respiratory bronchiole is seen above  $\times 260$

at the level of the respiratory bronchioles. No angiomas. A few larger arteries show medial hypertrophy and diffuso intimal hyperplasia without a duplication of the internal elastic lamina (fig. 10).

#### Case 5

Male, aged 32. Death due to pulmonary embolism.

*Autopsy* Heart (200 g) shows brown atrophy, no hypertrophy of right ventricle. Severe bilharziasis of intestine due to *S. mansoni* and of bladder and ureters from *S. haematobium*. Liver (1750 g) and pancreas cirrhotic and spleen enlarged (350 g).

*Lungs* contain *S. haematobium* ova only. no gross change.

*Histology* Majority of tubercles healed. There are a few embolic ova but no acute arteriolar lesions. A number of the arterioles are occluded or narrowed and there are a few small angiomas. Proximal to the occlusive lesions and angiomas the arterioles show medial hypertrophy.

#### Case 6

Male, aged 30. Patient died from double pyonephrosis secondary to bilharzial carcinoma of the bladder.

*Autopsy* Heart not hypertrophied (230 g). Liver (1100 g) shows brown atrophy but no cirrhosis. Spleen not enlarged (200 g). Colon slightly bilharzial. *S. haematobium* ova present in the ureters, bladder and prostate.

*Lungs* contain ova of *S. haematobium* only. no gross abnormality.

*Histology* There are numerous ova, mostly calcified, lying in old scars. Recent tubercles scanty. Embolic ova and acute arteriolitis absent. All vascular lesions apparently healed and consist of occluded arterioles and angiomas. The latter are more numerous, larger and older than in the other cases. There is no muscle in the wall, which consists of collagen enclosing several cavernous blood spaces (fig. 11), and the entering arteriole shows medial hypertrophy. Some of the larger muscular arteries are also hypertrophied.

#### Discussion.

Vascular lesions of the type illustrated by these cases are not described in the literature of schistosomiasis and constitute, we believe, a new observation. They are always associated with severe somatic bilharziasis, which provides the source for numerous ova to enter the pulmonary artery. Either *S. haematobium* or *S. mansoni* may be responsible. It is clear that emboli of ova must arrive at different periods as shown by the various ages of the lesions, and frequently they are in transit shortly before death. In some cases embolism has not occurred for some time, all the lesions being healed. Impaction of the ovum occurs at the level of the respiratory bronchiole, where the lumen of the artery is small enough to arrest it. The immediate effect is an acute necrotising arteriolitis with destruction of the intima and media in the vicinity. The ovum embeds itself in the swollen necrotic tissue, the cellular reaction is negligible and the breach in the intima is quickly covered by endothelium. Migration through the coats of the arteriole appears to be due to the necrotising action of the ovum, assisted no doubt by the thinness of the vessel wall, and it is only when an



## PLATE XXV

- FIG. 7—Case 1. Acute necrotising arteriolitis due to ovum of *S. mansoni*. Adjacent to the vessel is a bronchiole. The ovum is covered by swollen endothelium while the intima and media are converted into a hyaline necrotic mass. The adventitia is infiltrated with mononuclear cells. Note that the spine of the ovum points in the opposite direction to the line of egress. A giant cell representing part of an extravascular tubercle is seen on the right, distal to the vessel.  $\times 215$
- FIG. 8—Case 1. A large arteriole showing necrosis of the intima and media over which the swollen endothelium is unbroken. There is no thrombosis. The adventitia is infiltrated with cells. The ovum is not present in this section.  $\times 250$
- FIG. 9—Case 2. Obliterative arteriolitis. There is fibrocellular thickening of the intima and the same tissue fills the gap in the media at the upper pole of the vessel. In the adventitia is slight cellular infiltration. The ovum is not present as it has become extravascular.  $\times 200$

PULMONARY SCHISTOSOMIASIS



FIG 7

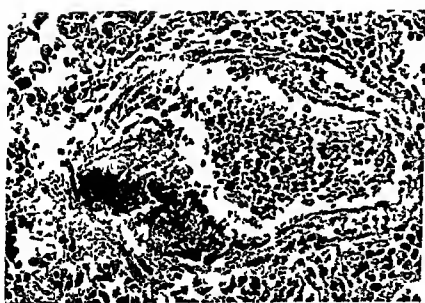


FIG 8



FIG 9





## Case 7.

Male, aged 18 Died with congestive heart failure, anasarca and ascites

*Autopsy* Old splenectomy scar. All viscera show chronic passive congestion Heart 430 g, right auricle and ventricle greatly dilated and hypertrophied, left ventricle 1.7 cm thick Main pulmonary artery and all valves normal Liver (1800 g) attached to diaphragm by vascular adhesions, advanced bilharzial cirrhosis Oesophageal veins varicose Pancreas cirrhotic Diffuse bilharzial infiltration of large intestine due to *S. mansoni*. Bladder, ureters and seminal vesicles slightly bilharzial Many ova in liver, pancreas, ureters, bladder and seminal vesicles

*Lungs* contain only *S. mansoni* ova

*Histology* No emphysema Although there is typical chronic passive congestion and recent escape of red cells into the alveoli the prussian blue reaction is not given Parenchymatous tubercles abundant the majority are recent but show very little necrosis The few calcified ova present are heavily impregnated with iron and enclosed in healed tubercles Neither embolic ova nor acute lesions are found in otherwise normal vessels The most striking changes are due to the arterial lesions, which are diffusely distributed, though some vessels have escaped infection Extensive obliterative arteriolitis, the lesions being in various stages of healing The causative ovum is rarely present, having migrated to an extravascular site However in some of these vessels there is evidence of reinfection,\* an ovum being embedded in the thickened intima, surrounded by polymorphs and leucocytes and producing a severe necrosis without formation of a tubercle The swelling produced by this reaction further reduces the lumen of the vessel Thrombosis absent. In other arterioles reinfection has led to complete occlusion of the lumen, which is filled with fibrocellular tissue canalised by capillaries while the media has entirely disappeared Proximal to this the vessel shows hypertrophy and where it enters the lesion the media ends abruptly Angiomatoids are numerous and mostly small, consisting of capillary-like spaces The larger (fig 14), which may attain 450  $\mu$  in diameter, are ovoid and composed of cavernous spaces between which is a young connective or argyrophil tissue At the periphery is a more mature connective tissue infiltrated with mononuclear cells and representing the original adventitia of the vessel Fragments of the media may persist between the cavernous spaces Proximal to the angiomatoid the artery is greatly hypertrophied but the media stops sharply at the point of entry, the elastica being reduplicated and frayed out here. Embolic ova may be present in the cavernous spaces and after fixation produce a necrotising lesion of reinfection in the surrounding tissue Owing to the diffuse distribution of the occlusive lesions there is widespread medial hypertrophy of the larger arteries with collagenous thickening of the adventitia in which there are no ova.

## Case 8

Female, aged 12, admitted with epigastric pain of six months' duration She died suddenly ten days later after a course of injections of "Fouadin" Clinically she presented a typical picture of congestive heart failure

*Autopsy.* Heart 290 g, marked dilatation and hypertrophy of right ventricle (1.0 cm.) which is thicker than the left ventricle (0.7 cm.) Chronic passive congestion of viscera No atheroma of pulmonary artery; all valves healthy Liver (1500 g) attached to diaphragm by vascular adhesions;

\* The reinfection is mainly if not exclusively endogenous, the source of the ova being the bilharziasis in other organs



PULMONARY SCHISTOSOMIASIS



FIG 10



FIG 11



FIG 12



FIG 13

## PLATE XXVI

- FIG 10—Case 4 Artery showing medial hypertrophy and intimal hyperplasia consecutive to occlusive lesions in its arteriolar branches Verhoeff and van Gieson  $\times 70$
- FIG 11—Case 6 An angiomatoid of cavernous type with fibrous walls devoid of muscle At its lower pole is the hypertrophied artery adjacent to which is a nest of *S haematobium* ova lying in scar tissue Above the ova is the bronchiole covered with cuboidal epithelium Iron hæmatoxylin and van Gieson  $\times 90$
- FIG 12—Case 9 *S mansoni* ovum free in the lumen of a thickened arteriole The wall consists of thickened intima and adventitia, the media has disappeared Iron hæmatoxylin and van Gieson  $\times 200$
- FIG 13—Case 9 A thickened arteriole in which there is no media, showing a necrotic lesion of reinfection due to an ovum of *S mansoni* Masson's trichrome stain  $\times 180$

## PULMONARY SCITE O-C 45.3

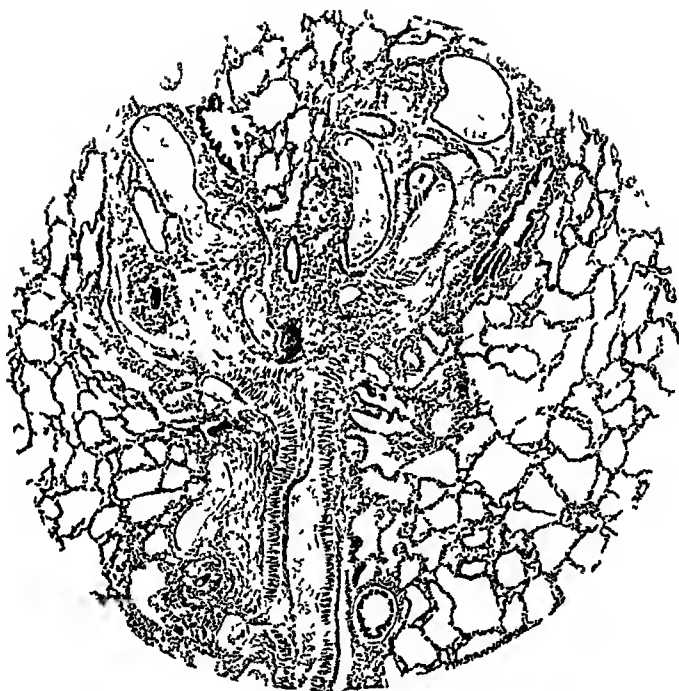


FIG 14—Case 7 In the upper half of the field are two cavernous angiomas which have developed in the branches of a hypertrophied artery In one angioma (right) are remnants of the media in the tissue of the other is a necrotic lesion of reinfection containing an ovum of *S. mansoni* In the left lower quadrant a small branch from the main artery is occluded by scar tissue at its origin and distal to this is canalised Close by is a bilharzial tubercle in the thickened adventitia Iron hematoxylin and van Gieson  
×45



cardium of left ventricle normal, of right diffusely white and opaque from subendothelial fibrosis. Intima of pulmonary artery and its two divisions diffusely white due to intimal fibrosis and covered by yellow nodules of atheroma. Aorta free from atheroma. Liver (1300 g) not cirrhotic, spleen not enlarged (160 g). Ureters and bladder show biliary infiltration. No gross intestinal lesions. Abundant ova (mostly calcified) of *S. haematobium* in bladder, ureters, seminal vesicles and intestine. No ova in liver.

**Lungs.** A pure infection with ova of *S. haematobium*. Organs very atheromatous and congested, no emphysema. In middle and lower lobes of right lung are two large hemorrhagic infarcts. Primary branches of pulmonary artery dilated and atheromatous and smaller divisions thickened and rigid, standing out like strands of silver wire (fig 19). No perivascular tubercles, nor could angiomatoids be detected, although microscopically they proved to be numerous and large.

**Histology.** The severe chronic passive congestion has not produced haemo-iderosis except for a few alveolar histiocytes giving slight diffuse staining. This case is essentially one in which the lesions are healed and the effects of prolonged vascular obstruction are in evidence. Apart from a few recent parenchymatous tubercles the numerous ova are nearly all calcified, impregnated with iron and enclosed either in parenchymatous scars or in the thickened adventitia of the arteries or the matrix of angiomatoids. The alveoli often contain dislodged ova. Most of the ova retain their shape but some are shrunken, distorted and fissured, suggesting dissolution.

There are no acute lesions and no evidence of recent reinfection, which agrees with the absence of embolic ova. The majority of the obstructed arteries have been converted into angiomatoids which vary greatly in size and maturity. The largest may attain 500  $\mu$  and may be entirely cavernous, though usually of mixed type with the capillary vessels in the centre and the cavernous at the periphery (fig 23). There is rarely a trace of muscle or elastic tissue present and the adventitia becomes continuous with the thickened outer coat of the hypertrophied artery. Frequently calcified ova are embedded between blood spaces and represent the end result of earlier reinfections. The absence of perivascular tubercles is due to healing, while the marked collagenous thickening of the adventitia explains the silver wire appearance of the arteries.

#### CASE 12

Male, 25 years of age. No particulars are available and the patient died on the second day after admission.

**Autopsy.** There is cyanosis and moderate oedema with excess of fluid in all the serous cavities. All viscera show extreme chronic passive congestion. Right heart very dilated and right ventricle (10 cm thick) hypertrophied (fig 24). In right auricular appendix are two mural thrombi. The left ventricle 12 cm thick. No valvular lesions. Circumference of pulmonary orifice 6.6, of aortic 5.2 cm. Aorta normal but main pulmonary artery shows some atheromatous plaques and microscopically there is diffuse fibrillar thickening of intima. Liver not cirrhotic and spleen not enlarged. Intestines show no biliary lesions and are negative for ova. Ureters thickened and narrowed by scar tissue in their distal half, producing a moderate degree of hydronephrosis. Bladder thickened and contracted, mucosa being smooth and rigid from scarring. Seminal vesicles enlarged, nodular and greatly thickened by scar tissue. Moderate number of *S. haematobium*-ova in bladder but only one seen in seminal vesicles and none in ureters. Ova all calcified. The appearances are typical of healed

bilharziasis but the extent and severity of the lesions are out of all proportion to the number of ova and evidently many of them have disappeared. This is significant as regards the pulmonary changes.

*Lungs* Several pieces of tissue were macerated but no ova found. In sections one calcified ovum was seen in an alveolus but was probably a contamination. Nevertheless the lesions are almost certainly due to *S. hæmatobrium*. On section, organs very congested and œdematous but no emphysema. Everywhere the branches of the pulmonary artery down to vessels of 2-3 mm are thickened, rigid and patulous from atheroma (fig 25). The smaller vessels appear as silvery white streaks. There are no perivascular tubercles and the angiomas are invisible.

*Histology* Chronic passive congestion marked, alveoli filled with fluid in which are some red cells. A few alveolar phagocytes contain traces of iron. This is the most advanced case in the series as the lesions are all healed and the ova have disappeared. The parenchymatous tubercles are reduced to scars and ova have not entered the lungs for a long time. The majority of the occluded arteries have been converted into angiomas, which constitute the end result of infection. The angiomas are more mature than in case 11, and larger, some being 0.5 to 1 mm in length. They consist of large cavernous spaces surrounded by old acellular collagen. Capillary spaces in a fibrocellular matrix are present in some of them, especially in the centre, but the conversion of these into the cavernous type can readily be traced (fig 26). The cavernous spaces are in free communication and thin branching fibrous spurs may project into the cavities, increasing the resemblance to true cavernous angioma. Thrombosis does not occur although conditions appear favourable. Usually the media of the artery has disappeared but fragments of muscle and elastica may persist, though, owing to progressive enlargement of the cavernous spaces, they now lie within the confines of the angioma. A diffuse fibrosis has occurred in the middle coat of the greatly hypertrophied arteries as the result of the prolonged obstruction to the circulation and the thickened adventitia has shrunk with maturation. The absence of ova could reasonably be advanced as a serious objection to the bilharzial origin of the disease were it not for the fact that in other organs healed lesions, undoubtedly bilharzial, occur in which it is extremely difficult or impossible to find ova. This is true to some extent of the urinary bilharzia in this case. Moreover the angiomas appear to be peculiar to pulmonary bilharziasis and at the autopsy four years ago, although we recognised the condition as Ayerza's disease, the angiomas were unfamiliar to us and their nature remained obscure until we met with bilharzial infection of the lungs in earlier stages.

### Discussion.

Although there are no statistics on the frequency of non-bilharzial Ayerza's disease the affection is an uncommon one, as MacCallum (1931) saw only one case in 12,000 necropsies. Our group constitutes 2.1 per cent of all cases of schistosomiasis and 6.3 per cent of the pulmonary cases, figures which are very high compared with the rarity of Ayerza's disease in other countries. In Egypt, schistosomiasis is obviously the most common ætiological agent, and the few case reports in the literature indicate that Ayerza's disease is probably a common complication wherever bilharziasis is endemic. Azmy Bey (1932) reported one case from

Cairo with atheroma of the pulmonary artery and endarteritis obliterans of the smaller vessels with ova in the walls, while Day (1936-37), in studying the disease from the clinical standpoint, confirmed the diagnosis in one case from material in our department. The disease has been recognised in Brazil (Silveira) and Clark and Graef (1935) described the obliterative arteriolar lesions in a Porto Rican. Only *S. mansoni* is endemic in Brazil and Porto Rico. Although pulmonary tubercles due to *S. japonicum* have been found by Faust and Meleney (1924) no cases of Ayerza's disease have been reported, but we feel this event may confidently be predicted as this parasite has a greater egg laying capacity than the other two schistosomes and produces lesions (Katayama disease) similar in distribution to *S. mansoni*. Either *S. haematobium* or *S. mansoni* can cause pulmonary vascular lesions (table II), although

TABLE II  
*Species of ova in 11 cases of bilharzial pulmonary arteritis*

Nature of lesion	Number of cases	<i>S. haematobium</i>	<i>S. mansoni</i>	Mixed
Focal arteritis	6	2	3	1
Ayerza's disease	5*	2	3	0
Total	11	4	6	1

\* (The no. 6 in which no ova were found was almost certainly due to *S. haematobium* but is not included.)

in cases of double somatic infection only one species may be present in the lungs. In none of the cases of Ayerza's disease had the condition been recognised during life. When first seen, all of them showed the signs of congestive heart failure. In cases infected with *S. mansoni* the splenomegaly and hepatic cirrhosis in conjunction with heart failure should suggest the possibility of pulmonary bilharziasis, but when the somatic and pulmonary disease are due to *S. haematobium* the case would present great difficulty in diagnosis.

From a pathological standpoint the group provides a fairly complete picture of the series of events from a relatively acute stage to complete healing. The gross appearances of the lungs are only specific when ova of reinfection fail to complete their migration through the wall of the thickened vessels and tubercles develop, producing perivascular nodules (fig. 18). At a later stage only the effects of long standing arterial obstruction remain, the thickened and atheromatous vessels presenting the same appearance as in non-bilharzial Ayerza's disease (figs. 19 and 24). Unfortunately





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the angiomas, which are a prominent feature in old cases, are invisible in our material

The atheroma of the pulmonary arteries and the hypertrophy of the right ventricle to 1.0 cm or more in thickness (and greatly in excess of that ordinarily produced by mitral disease) are a response to the increased intrapulmonary blood pressure and do not differ from other types of Ayerza's disease

The fact that the obstruction to the pulmonary circulation is on the arterial side of the capillaries has not prevented the occurrence of venous congestion. The lungs did not present the rust-brown colour of chronic venous congestion produced by obstruction to the outflow of venous blood, and although intra-alveolar hæmorrhage occurred there was either no hæmosiderosis or at most an inappreciable and recent deposit of iron

Microscopic changes in the pulmonary arteries in bilharzia were first briefly described by Miller (1914) but the only adequate account in the literature is one case of *S. mansoni* infection studied by Clark and Graef. They recognised the obliteration of the lumen by a richly vascularised tissue the result of canalisation without previous thrombosis, and the medial damage the cause of which, however, was obscure, owing no doubt to the absence of early lesions. The presence of angiomas is not mentioned. From our cases it is evident that the occlusive arteritis is the result of repair followed by persistent or intermittent reinfection superimposing new lesions on old. The massive infestation of the lungs is due, of course, to ova which have failed to become fixed in the somatic veins and affords impressive evidence of the imperfection of this process. After the death of the ova the specific characters of the lesions tend to disappear and eventually only angiomas remain. These may be regarded as adaptations to overcome the obstruction to the pulmonary circulation, and the growth in size of the blood spaces to cavernous dimensions beyond the confines of the original vessel must be greatly favoured by loss of the controlling effect of the media of the artery in which they develop. Although myohypertrophy of the larger arteries is induced by the oblitative arteritis, it progresses in spite of the development of angiomas. These structures appear to be peculiar to bilharzial arteritis, as they have not been described in other forms of Ayerza's disease. In non-bilharzial types of the disease cavernous spaces may occupy the lumen of the sclerosed arteries (Goedel, 1930; Rosenthal, 1930; Hora, 1934-35; Wiese, 1935-36), but as they are thrombotic in origin and lie inside an intact media they are quite distinct from bilharzial angiomas. Where all the ova have disappeared and with them their specific effects, as in case 12, the bilharzial origin of the disease rests upon the character of the lesions of the angiomas.

## HISTIOCYTOSIS



FIG. 15.—

right side  
A typical fibrous  
tissue  $\times 80$

Fig. 16.— A branch arising from the  
arteriole is containing capillaries  
and granules of the tissue



FIG. 17.—Case 9. A young angioma consisting of capillary and venous spaces arising from a hypertrophied artery the nuclei of which are at the entrance  $\times 70$





FIG 18—Case 10 Lung Typical appearance in bilharzial Ayerza's disease at a comparatively early stage. Miliary nodules are arranged in clusters or lines round the thickened arteries. Natural size

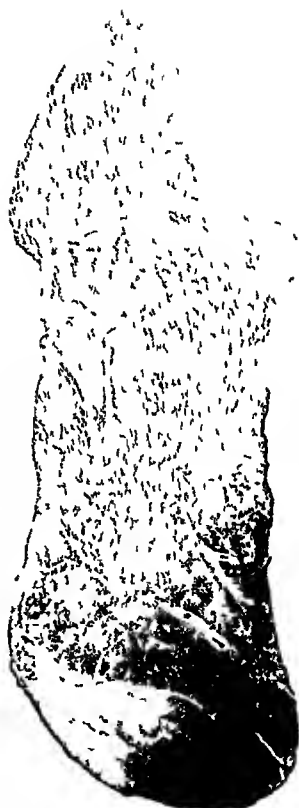


FIG 19—Case 11 Lung Bilharzial Ayerza's disease at a late stage. Note the silver wire appearance of the thickened arteries and the absence of miliary nodules.  $\times 1$

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## PULMONARY SCHISTOSOMIASIS



FIG. 20

ovum

lumen

artery

FIG. 21—C

of *S. mansoni* embedded in the wall of a thickened arteriole. It is covered by endothelium and the only evidence of necrosis is a slight blurring of the intima to left of the ovum.  $\times 220$

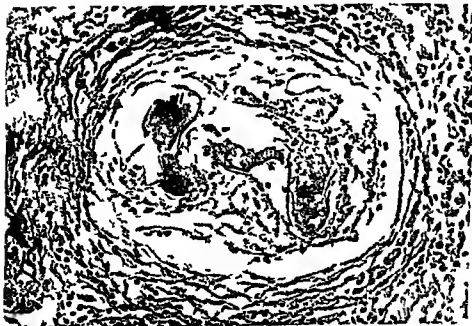


FIG. 22—Case 10. A canalised arteriole in which the media has disappeared. It contains three *S. mansoni* ova, two of which are lying free in the blood spaces while the third (to right) has become embedded in the wall, producing a very early lesion of reinfection as shown by necrosis in its vicinity.  $\times 220$

11-11-11





Fig. 1  
1  
nrls

Fig. 21 -  
of ...  
embedded in the wall  
a thickened arteriole. It  
is covered by endothelium  
and the only evidence of  
necrosis is a slight blurring  
of the intima to left of the  
ovum  $\times 210$



Fig. 22 - Case 10 - A canalised  
arteriole in which the media  
has disappeared. It con-  
tains three *S. mansoni* ova,  
two of which are lying free  
in the blood spaces while the  
third (to right) has become  
embedded in the wall pro-  
ducing a very early lesion  
of reinfection as shown by  
necrosis in its vicinity  
 $\times 220$



PULMONARY SCHISTOSOMIASIS



FIG. 23—Case 11. An artery with medial hypertrophy and hyperplastic intima opening into an arteriomatoid of mixed type in which are several calcified ova of *S. hematobium*. The media of the artery is not continued into the arteriomatoid. Iron hematoxylin and van Gieson.  $\times 120$ .

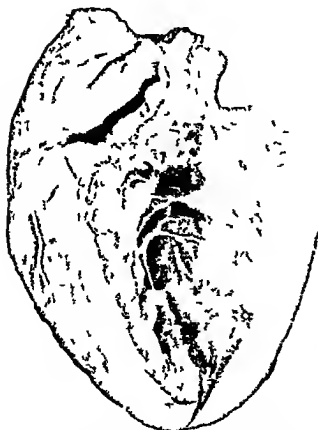


FIG. 24—Case 12. Heart showing dilation of the right auricle and great hypertrophy of the right ventricle.  $\times \frac{1}{2}$ .

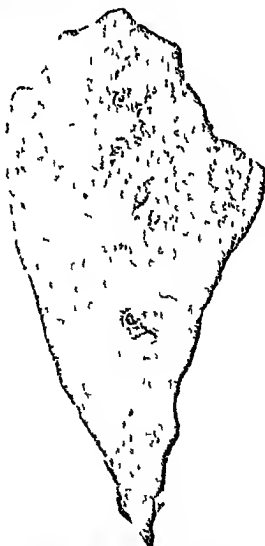


FIG. 25—Case 12. Lung showing atheromatous thickening of the arteries in long-standing bilateral Ayerza's disease.  $\times \frac{1}{2}$ .



### B Lesions due to worms

There are very few references in the literature to vermoid infection of the lungs in man. Worms were first found in the pulmonary blood of an Egyptian by Symmers (1905), who thought they were *S. haematobium*, although from what we now know they were almost certainly *S. mansoni*. Dew apparently detected none in his Egyptian autopsies and no further report appeared from this country until Day described a case in our laboratory with numerous coupled male and female worms of *S. mansoni* in the arteries. In Brazil, where only *S. mansoni* occurs, Silveira states that worms may be found in the pulmonary veins but, for reasons to be given, these were probably arteries. *S. japonicum* worms do not appear to have been found in the lungs although their presence is very probable.

In experimentally infected animals pulmonary involvement appears to be frequent. Fairley (1919-20) found *S. haematobium* and *S. mansoni* in monkeys and Faust and Meleney (1924) *S. japonicum* in the dog. In a monkey infected with *S. mansoni* we found many male worms in the pulmonary arteries.

In our material there were 10 cases with worms in the lungs, an incidence of 3.6 per cent in all cases of bilharziasis and 10.5 per cent in pulmonary infection. The actual incidence is certainly greater, as the area of lung tissue examined was very small. As the worms were present in arteries and never in veins their mode of entrance must be by migration or embolism. Apart from the fact that the former method involves a perilous journey through the right side of the heart and over the valves, the histological evidence is entirely in favour of embolism. They become impacted, usually as rider emboli, where the artery divides into two branches (fig. 27) too small to allow their further passage and the ends of the worms may trail into the branches. This position was clearly demonstrable in 7 cases (table III) though sometimes serial sections were necessary. Where two or more worms are lodged the vessel may be distended but there is never thrombosis or damage to the arterial wall while the worms are still alive (fig. 27).

Embolism occurred at different periods as shown by the presence of living and defunct worms. As many as five worms were found in one section but usually only one was present. Coupled male and female worms occurred once, in the other cases only males were present. As the species of worm cannot be identified in microscopic sections we have had to rely on the type of ova in the lungs and somatic lesions. It will be seen (table III) that both species of schistosomes may infect the lungs. In 9 cases all or some of the worms were defunct, showing that although bathed in venous blood conditions in the pulmonary arteries are

TABLE III.

Particulars relating to schistosome worms in the lungs in 10 cases.

Case no	Species	Number	Sex	State	Site in artery	Pneumonia	Ova in lungs	Lesions in lungs	Ova in other organs
1	?	2	Male	Living	Bifurcation		<i>S. mansoni</i> and <i>S. hæmatobium</i>	Focal arteritis	Unspecified ova intestines, liver (cirrhosis) Bladder negative
2	<i>S. mansoni</i>	5	Male and female	Living dead calcified	Bifurcation	Acute and healed	<i>S. mansoni</i>	Focal arteritis	<i>S. mansoni</i> peritonæum, liver (cirrhosis), pancreas Ureters, bladder, prostate negative
3	<i>S. hæmatobium</i>	1	Male	Calcified	Bifurcation	Healed	<i>S. hæmatobium</i>	Tubercles	<i>S. hæmatobium</i> - bladder, ureters, seminal vesicles Liver and intestines negative
4	?	1	Male	Dead	Bifurcation	Acute	<i>S. mansoni</i>	Tubercles	Unspecified ova intestines, liver (cirrhosis), pancreas, bladder, seminal vesicles
5	<i>S. mansoni</i>	2	Male	Dead	?	Acute and healed	<i>S. mansoni</i>	Ayerza's disease	<i>S. mansoni</i> - intestines, liver (cirrhosis), pancreas, peritoneum
6	<i>S. mansoni</i>	3	Male	Dead	Bifurcation	Acute and organising	<i>S. mansoni</i>	Tubercles	Ureters and bladder negative
7	?	1	Male	Dead	Bifurcation	Acute	<i>S. hæmatobium</i>	Focal arteritis	<i>S. mansoni</i> intestines, liver (cirrhosis) Bladder, ureters, seminal vesicles negative
8	?	1	Male	Dead	?	Acute	<i>S. hæmatobium</i>	Ayerza's disease	<i>S. mansoni</i> intestines seminal vesicles
9	<i>S. hæmatobium</i>	1	?	Calcified	?	Healed	<i>S. hæmatobium</i>	Tubercles	<i>S. hæmatobium</i> intestine bladder
10	<i>S. hæmatobium</i>	2	Male	Dead	Bifurcation	Acute	<i>S. hæmatobium</i>	Tubercles	<i>S. hæmatobium</i> bladder, prostate, seminal vesicles Intestine and liver negative
									<i>S. hæmatobium</i> intestine, bladder, ureters, seminal vesicles Liver negative

## PULMONARY SCHISTOSOMIASIS



FIG. 26.—  
Lung tissue  
vicina  
a hyp  
(lower  
Nata cor  
x100

FIG. 27.—Lung tissue  
and female *S. mansoni*  
worms impacted as a  
ridar embolus at the  
bifurcation of an ar  
tery x70



FIG. 28.—Dead *S. haematobium* worm impacted at  
bifurcation of an artery.  
The vessel wall is necrotic  
and the alveoli filled with  
a pneumonic exudate. A  
giant cell is seen at an  
end of the worm x80



TABLE III.

Particulars relating to schistosome worms in the lungs in 10 cases.

Case no.	Species	Number	Sex	State	Site in artery	Pneumonia	Ova in lungs	Lesions in lungs	Ova in other organs
1	?	2	Male	Living	Bifurcation		<i>S. mansoni</i> and <i>S. haematobium</i>	Local arteritis	Unspecified ova intestine, liver (cirrhosis) Bladder negative
2	<i>S. mansoni</i>	5	Male and female	Living, dead, calculated	Bifurcation	Acute and healed	<i>S. mansoni</i>	Local arteritis	<i>S. mansoni</i> peritonaeum, liver (cir- hosis), pancreas Ureters, bladder, prostate negative
3	<i>S. haematobium</i>	1	Male	Calculated	Bifurcation	Healed	<i>S. haematobium</i>	Tubercles	<i>S. haematobium</i> : bladder, ureters, seminal vesicles Liver and intes- tine negative
4	?	1	Male	Dead	Bifurcation	Acute	<i>S. mansoni</i>	Tubercles	Unspecified ova intestine, liver (cirrhosis), pancreas, bladder, semi- nal vesicles
5	<i>S. mansoni</i>	2	Male	Dead	?	Acute and healed	<i>S. mansoni</i>	Ayerza's disease	<i>S. mansoni</i> : intestines, liver (cir- hosis), pancreas, peritonaeum.
6	<i>S. mansoni</i>	3	Male	Dead	Bifurcation	Acute and organising	<i>S. mansoni</i>	Tubercles	Ureters and bladder negative <i>S. mansoni</i> intestines, liver (cir- hosis) Bladder, ureters, seminal vesicles negative
7	?	1	Male	Dead	Bifurcation	Acute	<i>S. haematobium</i>	Focal arteritis	<i>S. mansoni</i> : intestines <i>S. haematobium</i> bladder, ureters, seminal vesicles
8	?	1	Male	Dead	?	Acute	<i>S. haematobium</i>	Ayerza's disease	<i>S. mansoni</i> intestino <i>S. haematobium</i> bladder
9	<i>S. haematobium</i>	1	?	Calculated	?	Healed	<i>S. haematobium</i>	Tubercles	<i>S. haematobium</i> : bladder, prostate, seminal vesicles Intestine and liver negative
10	<i>S. haematobium</i>	2	Male	Dead	Bifurcation	Acute	<i>S. haematobium</i>	Tubercles	<i>S. haematobium</i> intestine, bladder, ureters, seminal vesicles Liver negative



## PULMONARY SCHISTOSOMIASIS



FIG. 2.—*S. haematobium* worms in the lungs (lower)  $\times 100$

FIG. 27.—Living male and female *S. haematobium* worms impacted in a rider embolus at the bifurcation of an artery  $\times 70$



FIG. 28.—Dead *S. haematobium* worm impacted at the bifurcation of an artery. The vessel wall is thickened and the alveoli filled with a pneumonic exudate. A giant cell is seen at one end of the worm  $\times 80$



unfavourable to survival. While the living worm is harmless the products of the dead parasite are extremely toxic, producing a characteristic necrotic and focal pneumonia which appears as an opaque white round or elliptical area of consolidation 0.1-0.5 cm in size with the worm in the centre. Death of the worm is followed by thrombosis. The vessel wall quickly becomes necrotic and invaded by eosinophil leucocytes and some histiocytes, the latter sometimes forming giant cells which attack the worm (fig. 28). The walls of the surrounding alveoli are congested and the lumen filled with fluid, fibrin, many eosinophils, some polymorphs and histiocytes and a few red cells. Outside the pneumonic area there may be intra-alveolar haemorrhage. With diffusion of the toxin the inflammatory exudate and alveolar walls also become necrotic, the arterial wall disappears and the dead worm is now extravascular (fig. 29). The area of necrosis is reduced to a dense structureless mass, at the periphery of which the pneumonic exudate organises (fig. 30). Later the necrotic debris is absorbed but the tissues are incapable of autolysing the dead worm, which calcifies and is enclosed in a capsule of scar tissue (fig. 31).

In half the cases with vermoid infection there were focal or diffuse vascular lesions but in the remainder parenchymatous tubercles only (table III). The early stages of worm pneumonia were first described in experimentally infected monkeys by Farley (1919-20) but do not appear to have been recognised in man hitherto.

#### THE ROUTE TRAVERSED BY THE PARASITES TO THE LUNGS

As the vesical, ureteric, prostatic and uterine venous plexuses drain into the internal iliac veins, detached ova and worms of *S. haematobium* can readily enter the lungs\*. This explains to some extent why this species is found there more frequently than *S. mansoni* (table I) and also why pulmonary infection can occur when the urinary tract lesions are recent or slight. Nevertheless it will be seen that *S. haematobium* produces focal and diffuse vascular disease significantly less frequently than *S. mansoni* (table IV).

The habitat of *S. mansoni* is the portal area, between which and the systemic veins are anastomoses which normally seem insufficient to allow the passage of many parasites. For reasons already stated it is unlikely that ova or worms can traverse the liver and we have never found microscopic evidence of this. In *S. mansoni* infection of the lungs hepatic cirrhosis and severe somatic lesions are almost invariably present, indicating a massive infestation (table IV). The progressive obstruction in the portal system leads to hyperplasia of the collateral circulation as shown

\* In rare instances *S. mansoni* infects the genito-urinary area.

TABLE IV.

*Illustrating the relation between somatic and pulmonary lesions and the species of ova in the lungs in 32 cases of schistosomiasis*

Species of ova in lungs	No of cases	Severe somatic lesions	Cirrhosis of liver	Pulmonary arteritis
<i>S. hæmatobium</i>	21	12 (57 per cent)	2 (8 per cent) *	4 (19 per cent)
<i>S. mansoni</i>	11	10 (91 per cent)	10 (91 per cent)	6 (54 per cent)

\* Due to *S. mansoni* infection

by the vascular adhesions between the liver and diaphragm and the varicosity of the œsophageal veins. It is only, then, at a comparatively late stage of the disease that conditions are favourable for pulmonary infection, which will tend to be massive and therefore productive of arterial lesions. Day, from the clinical standpoint, has drawn attention to the association between cardio-pulmonary symptoms in bilharziasis and splenomegaly and hepatic cirrhosis, but erroneously concluded that pulmonary disease with clinical symptoms is always due to *S. mansoni* infection. This can readily be understood, as in pulmonary arteritis due to *S. hæmatobium* there is no cirrhosis or portal obstruction unless of course there is a double somatic infection.

#### THE MODE OF ESCAPE OF OVA FROM THE VESSELS.

The generally accepted view is based upon the work of Fairley (1919-20) on monkeys experimentally infected with *S. hæmatobium* and *S. mansoni*, whose mesentery had been exposed under anaesthesia. He observed that the female worm, leaving her partner, migrates by means of her suckers until she has stretched the venule to its uttermost. The ovum is deposited with its spine directed posteriorly. The worm then withdraws slightly and the vein contracts on the ovum so that the spine engages in the vessel wall and pierces it, while the force of the venous blood stream tears a rent in the venule and drives the ovum into the perivenous tissue. The actual transit of the ovum through the vessel wall was seen by direct microscopic examination of pieces of small intestine squeezed between two glass slides by means of rubber bands.

There was apparently no hæmorrhage and from the nature of the experiments necrotic or other changes in the vessel would be invisible. As regards embolic ova in the lungs and other organs Fairley (p. 294) says, "Escape into the perivascular tissues in these organs is produced by mechanical rupture by ova impacted

TERTIARY SCHISTOSOMIASIS



FIG 29—The dead worm is seen in the bifurcation of an artery whose walls have become thickened. The surrounding alveolar walls and pneumonic exudate are also visible.



FIG 30—Showing late necrosis of the pneumonic lung with organization of the inflammatory exudate in the peripheral alveoli. The dead worm is not shown.  
x110





2 The toxic effect of the ovum is shown by necrosis of the tissue in its vicinity. The amount of necrosis varies and possibly depends on the degree of allergy at the time of invasion.

3 The tissues may respond to the presence of ova by other reactions than the formation of tubercles and it is suggested on the histological evidence that the number of ova, reinfection, immunity and allergy may all play a part in determining the type of response.

4 The ova reach the lungs as emboli and become impacted in the arterioles which accompany the respiratory bronchioles, producing a specific acute necrotising arteriolitis. Following necrosis the ovum escapes through the vessel wall and a parenchymatous tubercle forms near the respiratory bronchiole. It is suggested that the ovum secretes an anticoagulant, as it never excites thrombosis.

5 The number of ova is of primary importance in deciding the effect on the pulmonary tissue. In 86 per cent of cases only a few ova had entered the lungs and the only lesions present were parenchymatous tubercles. Embolic ova are rarely seen in these cases.

6 In cases with a heavier infestation vascular lesions as well as parenchymatous tubercles are present and embolic ova are frequent. Healing of the acute vascular lesions leads to an obliterative arteriolitis, often followed by canalisation of the occluding tissue. The new-formed capillaries hypertrophy, producing a structure characteristic of pulmonary bilharziasis to which we have given the name "angiomatoid". The vascular changes are focal in distribution and are unassociated with cardiac hypertrophy or signs of congestive heart failure.

7 Massive and repeated infection of the lungs is followed by widespread arterial changes, hypertrophy of the right ventricle and the development of the cardio-pulmonary features of Ayerza's disease with death from congestive heart failure. The severity of the disease is largely due to repeated reinfection of healing or healed lesions. The gross appearances of the lungs are only specific when ova of reinfection fail to complete their migration through the thickened arterioles and tubercles develop in the walls. At a later stage, when the specific lesions heal, only the effects of long-standing arterial obstruction are evident and the naked-eye appearances do not differ from those in Ayerza's disease due to other causes. Microscopically, however, the bilharzial origin can be recognised by the characteristic angiomatoid structure, even although all the ova may have disappeared. The lungs are the seat of chronic passive congestion without hæmosiderosis.

8 Ayerza's disease of bilharzial origin comprises 2.1 per cent of all cases of schistosomiasis and 6.3 per cent of the pulmonary cases. Reasons are given for believing that it is a common



PULMONARY SCHISTOSOMIASIS



FIG 31—A completely disorganised and partially calcified *S. haematobium* ovum surrounded by scar tissue  $\times 60$

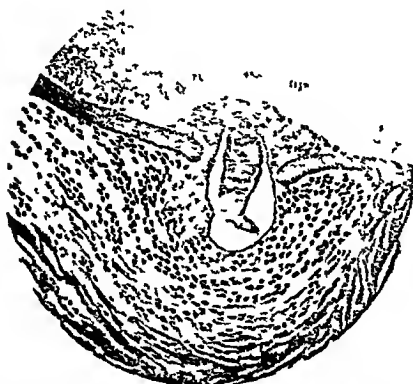


FIG 32—Case 9 Showing an ovum of *S. mansoni* passing through the fibrous wall of a large cavernous space in an angiomatoid. The toxic action of the ovum is shown by the altered staining reaction of the collagen and necrosis of the inflammatory cells in the intima and adventitia. Iron haematoxylin and van Gieson  $\times 200$

2 The toxic effect of the ovum is shown by necrosis of the tissue in its vicinity. The amount of necrosis varies and possibly depends on the degree of allergy at the time of invasion.

3 The tissues may respond to the presence of ova by other reactions than the formation of tubercles and it is suggested on the histological evidence that the number of ova, reinfection, immunity and allergy may all play a part in determining the type of response.

4 The ova reach the lungs as emboli and become impacted in the arterioles which accompany the respiratory bronchioles, producing a specific acute necrotising arteriolitis. Following necrosis the ovum escapes through the vessel wall and a parenchymatous tubercle forms near the respiratory bronchiole. It is suggested that the ovum secretes an anticoagulant, as it never excites thrombosis.

5 The number of ova is of primary importance in deciding the effect on the pulmonary tissue. In 86 per cent of cases only a few ova had entered the lungs and the only lesions present were parenchymatous tubercles. Embolic ova are rarely seen in these cases.

6 In cases with a heavier infestation vascular lesions as well as parenchymatous tubercles are present and embolic ova are frequent. Healing of the acute vascular lesions leads to an obliterative arteriolitis, often followed by canalisation of the occluding tissue. The new-formed capillaries hypertrophy, producing a structure characteristic of pulmonary bilharziasis to which we have given the name "angiomatoid". The vascular changes are focal in distribution and are unassociated with cardiac hypertrophy or signs of congestive heart failure.

7 Massive and repeated infection of the lungs is followed by widespread arterial changes, hypertrophy of the right ventricle and the development of the cardio-pulmonary features of Ayerza's disease with death from congestive heart failure. The severity of the disease is largely due to repeated reinfection of healing or healed lesions. The gross appearances of the lungs are only specific when ova of reinfection fail to complete their migration through the thickened arterioles and tubercles develop in the walls. At a later stage, when the specific lesions heal, only the effects of long-standing arterial obstruction are evident and the naked-eye appearances do not differ from those in Ayerza's disease due to other causes. Microscopically, however, the bilharzial origin can be recognised by the characteristic angiomatoid structure, even although all the ova may have disappeared. The lungs are the seat of chronic passive congestion without hæmosiderosis.

8 Ayerza's disease of bilharzial origin comprises 2.1 per cent of all cases of schistosomiasis and 6.3 per cent of the pulmonary cases. Reasons are given for believing that it is a common

## PULMONARY SCHISTOSOMIASIS



FIG 31 —A completely disorganised and partially calcified *S. haematobium* worm surrounded by scar tissue  $\times 60$

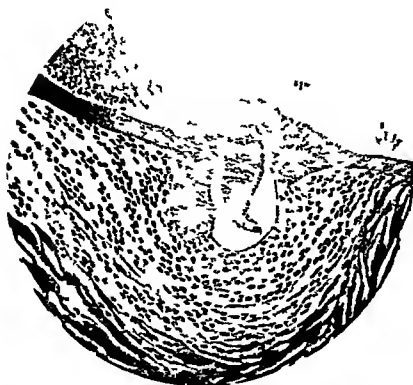


FIG 32 —Case 9 Showing an ovum of *S. mansoni* passing through the fibrous wall of a large cavernous space in an angiomatoid. The toxic action of the ovum is shown by the altered staining reaction of the collagen and necrosis of the inflammatory cells in the intima and adventitia. Iron haematexylin and von Gieson  $\times 200$



complication in Egypt and other countries where bilharziasis is endemic

9 Ova of *S. haematobium* (58 per cent) are more common in the lungs than *S. mansoni* (31 per cent, table I), but *S. mansoni* (54 per cent) produces vascular lesions more often than *S. haematobium* (19 per cent, table IV) The reasons for this are discussed

10 Evidence is advanced to show that the passage of the ovum through the vessel wall is due to necrosis produced by a toxic action of the embryo, the spine playing little or no part in the process It has also been shown that the ovum can escape through the wall under conditions where the size of the vessel and the absence of contractility make it impossible for the spine to exercise the piercing function attributed to it by the mechanical theory

11 Worms were present in the lungs in 3.6 per cent of the series and in 10.5 per cent of the pulmonary cases Either *S. haematobium* or *S. mansoni* may occur

12 The worms reach the lungs by the pulmonary artery and are usually arrested as riding emboli at the bifurcation of a vessel Although bathed in venous blood they rapidly die While alive they produce no structural changes, but the dead worm is highly toxic, causing necrosis of the artery and an acute focal necrotising pneumonia Later the pneumonic exudate is absorbed and cicatrised but the defunct worm becomes calcified and enveloped in scar tissue

We wish to express our cordial thanks to Mr N Streckalovsky, artist to the Faculty of Medicine, Cairo, who made the coloured drawings, and to Bakr Secoudy Effendi for the photomicrographs

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formation of large cytoplasmic acidophile inclusion bodies. Moreover the latter have been shown by Lipschutz and by von Prowazek to contain innumerable small granules which are now accepted as typical elementary bodies resembling those found in vaccinia by Buist in 1887 (see Gordon, 1937, Mackie and van Rooyen, 1937) and in other virus diseases.

Goodpasture and King (1927) have reinvestigated the development of the molluscum inclusion body by means of stained sections and smear preparations and have arrived at the following conclusions:

'The main factor in the hyalinization of the intracellular mass is desiccation. The hyaline oval masses formed by a coalescence of granules and cytoplasm constitute the mature "molluscum bodies" of Henderson and Paterson. They are not formed by a sort of keratinization as suggested by Lipschutz, but by fusion and desiccation of the elementary bodies and the intervening cytoplasm.

'It is evident from our preparations that the particles which appear to be extruded from the nucleus have no part in the composition of the elementary bodies, but dissolve and become a part of the basophilic cytoplasm which has a central position within the cell. It is within this altered cytoplasm that the vacuoles develop, about which and eventually within which tiny bodies occur having no counterpart among the cellular constituents. Mitochondria play no part in the formation of the elementary bodies. In fact the elementary bodies constitute, from all cytologic appearances, a new substance which increases enormously in bulk in the affected cells, and not by accretions to the size of individual bodies, but by a proliferation of innumerable bodies of uniform size, form, and staining qualities. The cytologic changes are in every way consistent with an active growth of a very minute living microorganism. Smear preparations, made by stroking a glass slide over the pearly core of a lesion moistened with physiologic salt solution, and suitably stained, confirm the cytologic appearances of an intracellular microorganism.'

According to these authors, then, the formation of the molluscum body is effected by fusion and desiccation of the elementary bodies and intervening cytoplasm of the affected cell. The process moreover takes place without the formation of a membrane covering the inclusion body.

Findlay in his excellent and comprehensive review of the literature of this subject, makes the following further reference to the absence of a membrane encircling the molluscum body:

"The development of these molluscum bodies has recently been reinvestigated by Goodpasture and King (1927), whose description corresponds very closely with the account of the development of the virus inclusions in fowl-pox given by Ludford and Findlay (1926), except that in human molluscum contagiosum the virus vacuoles do not acquire a lipid coat."

#### THE PRESENT INVESTIGATION.

The work was carried out with material obtained from ten cases of molluscum contagiosum under treatment by Drs Percival and





possible to bring the optical axis of the microscope over the dissecting needles with ease. Thus both the needle points could be centred simultaneously without touching the micromanipulating stands, or moving the film of cells (above the needles) placed on the dissecting chamber coverslip. The latter was controlled by the mechanical stage.

*Dissecting chamber* This was constructed from glass according to the author's own requirement and was not of standard pattern. It consisted of a glass slide 76 mm long, 37 mm wide and 1.1 mm thick, on which was cemented together a rectangular glass chamber 20 × 30 mm constructed of two sides 35 mm long, 7 mm deep and 4 mm wide and two lateral walls 20 mm long, 2.5 mm deep and 2.5 mm in width.

Two glass "troughs" were also provided along the lateral border of the chamber into which water was placed in order to maintain a sufficient degree of moisture within the chamber during dissection.

### *Technique*

Glass coverslips 38 mm long, 31 mm broad and 0.14 mm thick were cleaned by boiling in a mixture of potassium dichromate solution and sulphuric acid, washed with water, placed in methylated spirit for 1 hour, removed and dried with a silk cloth. The surface of the coverslip was then treated with a few flakes of tilarum which was rubbed into the glass with an artist's paper pencil. The coverslip was subsequently passed rapidly through the bunsen flame three times, placed on the laboratory bench and the excess of grease wiped off its surface with a silk rag.

A fresh moist fragment of molluscum tissue about 1 mm in diameter was now placed at one end of the coverslip and uniformly spread over the surface by crushing it with the edge of a 3 in × 1 in glass slide.

While the smear was still moist one drop of a 1:10,000 dilution of brilliant cresyl blue in 0.86 per cent saline was placed on the film and gently allowed to flow over it. The coverslip was next picked off the laboratory bench and the film placed inverted over the dissecting chamber. Next both manipulating needles were accurately centred in the field so as to take up a position slightly below the level of the film without actually touching it. Both lateral openings through which the needles passed into the dissecting chamber were now sealed up with vaseline and the interior of the cavity made air-tight. Any minor adjustments to the position of the substage condenser, iris diaphragm and reflecting mirror were made at the same time.

Prior to commencing micromanipulation the  $\frac{3}{4}$  in objective and  $\times 10$  ocular were placed in position, the light adjusted to maximum intensity and a suitably stained group of molluscum bodies manoeuvred into the centre of the field. At this stage both dissecting needles should be lying about 250  $\mu$  below the level of the film and should not be permitted to establish contact with the wet surface of the film. The needles were next elevated vertically until the point of each became just recognisable as a blurred image in the field.

To facilitate the latter operation the N.A. of the condenser was decreased by closing the iris diaphragm slightly, in order to give greater depth of focus and to render both needle points and cells lying above them more easily visible. The needle points, which should be close to each other, were re-centred in the field and the cell or group of cells to be dissected moved so that they lay immediately above the needles. The 6L (Leitz) objective and  $\times 10$  eyepiece (total magnification 150) were now substituted for the others and the dissecting needles gently raised and adjusted so that one needle lay immediately beneath the cytoplasm and the other below the inclusion body (fig. 2). A drop of cedar-wood oil was placed on the coverslip,







*Injection of carbon particles into the inclusion cavity*

The indian ink used was first filtered and then centrifuged at 3000 *r.p.m.* for 30 minutes. After withdrawing the inclusion body from an epithelial cell, the sharp-pointed needle was discarded and there was substituted a hollow glass pipette possessing a lumen of about  $2\ \mu$  and an external diameter of about  $2.4\ \mu$ . The pipette, filled with indian ink, was inserted into the cavity and the ink discharged into it. By this method it was possible to fill the cavity with carbon particles and to demonstrate that there was no tendency for them to flow into the adjacent cytoplasm. By rupturing the cavity wall, however, the ink was made to flow into it. The boundaries of the cavity were also delineated by injecting indian ink into the cytoplasm so that the ink outlined the margins of the cavity but did not enter it—unless it was punctured with a needle point either before or after introducing the ink.

*Observations with dark-ground illumination*

When an epithelial cell containing an inclusion body was examined under dark-ground illumination, both the cytoplasm and the molluscum body appeared to be full of refractile particles, but when the inclusion was removed from the cell, a vacant semi-transparent space was left where previously the inclusion body had lain. Again there was no evidence of a lining membrane.

The refractile granules within the cytoplasm were closely compared with those inside the molluscum body and a number of significant differences were detected. From their histological studies Goodpasture and King concluded that the epithelial cell contains myriads of elementary bodies measuring about  $0.25\ \mu$  in diameter. The writer, however, believes that the majority of the refractile particles evident within the cytoplasm of the epithelial cell are probably minute vacuoles or protoplasmic granules and not true elementary bodies. His reasons for this assumption are the fact that the refractile particles in the cytoplasm range from  $0.5$  to  $3.5\ \mu$  in diameter, whereas the stained molluscum elementary body is remarkably uniform in size— $0.3\ \mu$  by mensuration and  $0.35\ \mu$  when estimated by micrometric extinction (van Rooyen, 1937). By exerting pressure on the cell and exploring its contents by inserting a needle, many of the refractile spots in the cytoplasm could be shown to be minute spaces surrounded by concentric diffraction rings.

Similar examination of the molluscum inclusion body showed that its structure was entirely different, for it contained numerous refractile particles which in shape, size and degree of refractivity were identical with typical virus elementary bodies (fig. 11). Some additional evidence in support of this contention was derived from



against the flattened edge of a micro-spatula and a sharp needle was pushed into the opposite side the needle bent before puncturing the cell (fig 14)

2 The rounded, blunt extremity of the inclusion body appeared to have the thickest wall and when the cell was compressed in its long axis the thin-walled cap tended to flatten out against the micro-spatula as pressure was applied with the needle from the blunt end. thereafter the needle point bent and finally the cell wall yielded inwards

3. When this procedure was reversed and the flat edge of the micro-spatula was applied to the blunt extremity and the needle point placed against the cap a different sequence of events was observed when force was exerted. The needle point immediately and easily penetrated the thin cap of the inclusion body whilst the thicker, broader segment remained firm and rigid against the face of the spatula (fig 15)

4 If the inclusion body was picked up between the points of two needles and allowed to rotate between them the presence of the cap could be detected as the inclusion body altered its axis in the field

5 By compressing the inclusion body against the under side of the coverslip with a broad needle about  $10\ \mu$  in diameter the cap could be caused to bulge (fig 13) and finally to burst. By gradually increasing the pressure the contents of the body were forced out through the breach

6 When the inclusion body was transfixed at either pole with a sharp pair of needles and then pulled apart (fig 16) the low tensile strength of the cap was immediately apparent. Shortly before the body was torn asunder it was noticed that the line of cleavage first appeared at the conical pole and later the cell threatened to divide at its uppermost portion. The membrane of the lower (broader) segment tended to remain rigid and usually resisted tearing when subjected to stretching in this manner.

Thus after a series of optical and mechanical tests performed on the molluscan inclusion body the evidence proved the existence of thin-walled and thicker-walled poles. It should be clearly understood, however, that this only applies to bodies which were oval or pyriform in contour. The smaller circular inclusions appeared to possess a wall of uniform thickness. There is no evidence to show whether the size and shape of these bodies is related to their age and degree of development, and if so, which of the two represents the later stage of growth

#### *The contents of the molluscan body.*

Before measuring the wall of the inclusion body it was photographed by dark ground illumination and found to contain numerous











MICRODISSECTION OF MOLLUSCUM BODY



FIG 12



FIG 13



FIG 14



FIG 15



FIG 16

## PLATE XXXIX

- FIG 12 —Film from a molluscum lesion stained by Paschen's method for elementary bodies. Observe the enormous number of these minute virus bodies, also the large molluscum inclusion body in the left upper quadrant  $\times 1200$
- FIG 13 —Effect of compressing a molluscum body with a micro-needle against a coverslip. The needle is pointing obliquely upwards. The contents tend to herniate through the conical pole, thus revealing its structural weakness  $\times 1100$
- FIG 14 —Demonstrates the mechanical strength of the lateral walls of the molluscum body by compressing it between the sharp point of a needle and a flat micro spatula. Note the lighter staining "cap" or tapering pole  $\times 1100$
- FIG 15 —Reveals the ease with which the needle point can be inserted into the interior of the molluscum body through the weak conical pole. The membrane at this point offers no resistance to the pressure of the needle  $\times 1100$
- FIG 16 —Two micro needles have transfixed a molluscum body at either extremity and then been pulled apart. Observe the low tensile strength of the thin wall of the conical pole and its tendency to break  $\times 1100$

MICRODISSECTION OF MOLLUSCUM BODY



FIG 17—Shows the internal contents of a molluscum body in process of removal with the point of a dissecting needle. The contained material tends to be mucoid in consistency and adheres to the point of the needle. The minute elementary bodies are only just visible in the gelatinous matrix surrounding them. After removing this jelly like substance the empty "shell" of the molluscum body loses its former segmented internal appearance. 1100



FIG 18—Film stained by Paschen's method showing numerous molluscum elementary bodies which have been liberated from a ruptured inclusion body.  $\times 1100$





refractile bodies closely resembling in shape, size and degree of refractivity the Paschen bodies of vaccinia and other virus diseases. These elementary bodies were not easily photographed *in situ*, for the surface of the living molluscum body is curved, it possesses a membrane of varying thickness and, as will be referred to later, the elementary bodies are embedded in a fluid matrix. Fig 11 shows four molluscum bodies containing elementary bodies in their interior. These are necessarily indistinct because the elementary bodies lying immediately above and below the optimum plane of focus tend to introduce shadows into the field and impair the crispness of the images in focus. When stained by intra-vital dyes such as brilliant cresyl blue, the molluscum body presented a mottled appearance as though it were segmented internally into a series of compartments. von Prowazek (1911) and Goodpasture and King (1927) have depicted this appearance but make no suggestion as to its nature. Later an explanation of this appearance will be offered.

The dissection of the molluscum body was proceeded with by first transfixing it with one sharp needle and then incising the cell membrane with the other (figs 9 and 10). The body cavity was then explored by passing the "tearing" needle into its interior and manoeuvring it in such a manner as to facilitate the escape of the contents. Careful examination of the escaping material showed it to consist of innumerable elementary bodies, apparently held together by a gelatinous, mucoid material which slowly diffused throughout the surrounding fluid (figs 17 and 18). This jelly like material readily adhered to the needle points.

The markings on the molluscum inclusion body were therefore believed to be due to an optical effect, the result of the passage of light through a globoid, semi translucent envelope filled with viscid fluid throughout which numerous dense, highly refractile particles were dispersed. The appearance of compartments and whorls arose, in the writer's opinion, from varying degrees of density in the gelatinous fluid within the inclusion body and conceivably also from the effect of unevenly dispersed elementary bodies within that material.

Fig 12 shows a smear made from a molluscum lesion stained by Paschen's method for elementary bodies. Numerous elementary bodies are seen in close proximity to a ruptured molluscum inclusion body.

#### DISCUSSION

The inclusion body of molluscum contagiosum is generally regarded as a typical example of the inclusion bodies found in virus diseases, but from the present studies it would be incorrect to assume that this is so. The methods of investigation here described have not yet been extended to other virus diseases and until this

is done, the present findings must necessarily refer only to the molluscum inclusion body. It remains to be seen whether or not further micromanipulation studies will reveal that the structure of other virus inclusions, such as the Bollinger, Guarneri and Marchal bodies, is identical with that of the molluscum body.

Goodpasture and Woodruff (1931) have already carried out comparative micromanipulation studies on the Bollinger body and the molluscum body. They found that the molluscum body could be freed from the surrounding epithelial cells by tryptic digestion, but that it then became sticky and gelatinous so that it could not be manipulated readily with the Chambers microdissection apparatus.

The writer has likewise failed to microdissect with the Chambers apparatus molluscum inclusion bodies which had first been digested with trypsin. By means of the Janse-Péterfi type of instrument, however, and the use of fresh tissues stained *intra vitam* with brilliant cresyl blue, it was possible to examine the molluscum body under natural conditions and to preserve those finer morphological details which are unavoidably lost in digested tissues. This work has confirmed earlier knowledge of the molluscum body with regard to the elementary bodies contained within it and has also drawn attention to additional facts concerning its structure. Thus it has been shown that the inclusion body can be extracted from the epithelial cell without disintegration of the latter. There is no evidence of any connection between the two and the lining of the cavity which remains appears to be formed by a localised protoplasmic condensation which can be punctured and broken down with a needle.

Goodpasture and King have suggested that the main factor responsible for the production of the molluscum body is desiccation and fusion of smaller vacuoles within the cytoplasm of the infected cell. The writer can find no evidence which supports this view.

Indeed there is no evidence that the cytoplasm in any way participates in the composition of the molluscum body. About one hundred inclusion bodies have been examined, and every one from the smallest to the largest has appeared as a totally independent structure within the cell. The writer believes that the inclusion grows from the elementary body after it enters the cell. He does not believe that it is formed from the vacuoles so commonly seen in the parasitised epithelial cell. These are more probably formed as a result of degenerative changes due to interference with cell metabolism by the growth of the parasite within it. It is also conceivable that many of these vacuoles may represent elementary bodies which have been destroyed by intracellular ferments.

The pear-shaped contour of most of the molluscum bodies and the semi-translucent "cap" at the conical extremity are of considerable interest. This cap is demonstrably the thinnest part

of the body wall and rupture with liberation of the contained elementary bodies can be more easily effected here than at any other point

It is suggested that the large pear-shaped molluscum body represents the most fully advanced developmental stage of the virus, in contradistinction to the smaller spherical inclusions which have a membrane of uniform thickness surrounding them

Whether or not the possession of a thin-walled conical pole should entitle the molluscum inclusion body to be placed in a category separate from the inclusions of other viruses cannot be determined. From the evidence presented it is interesting to record that in some respects, in miniature, the molluscum body resembles the general structure of a fungus sporangium. Further work on this aspect of the subject is in progress

### CONCLUSIONS

1 Fresh material obtained from ten cases of human molluscum contagiosum has been stained *intra vitam* with brilliant cresyl blue and subjected to microdissection

2 The molluscum inclusion body has been successfully removed from the infected epithelial cell. It has also been replaced in the same cell without injuring the latter

3 The removal of the molluscum body leaves a large cavity in the cell. The cytoplasm does not flow into this space, the cavity being apparently lined by a localised condensed layer of cytoplasm

4 The boundaries of this cavity have been defined by injecting indian ink into it or alternatively by introducing indian ink into the surrounding cytoplasm

5 About one hundred molluscum bodies from the smallest to the largest have been removed from the containing epithelial cells by microdissection and in no case was there any evidence of attachment between body and cytoplasm

6 The molluscum body probably grows from a more minute form of the virus. It is not formed by desiccation nor by fusion of any intracytoplasmic constituents of the cell

7 It is suggested that the large oval or pear shaped forms of the molluscum body represent a later stage of development than the smaller spherical ones

8 The large pear-shaped inclusions have been shown to possess a definite outer membrane which is thickest at the conical pole. These observations have been established by demonstrating the tensile strength of the membrane on "palpation" with the point of a micro needle. The weakness of the conical segment has also been revealed by transfixing the inclusion body between two needles and exerting traction on its walls

9 The elementary virus bodies contained within the molluscum inclusion body have been shown to be supported in a gelatinous mucoid substance

I desire to express my indebtedness to Professor Mackie, at whose instigation this research was carried out and to whom I am indebted for much constructive criticism and help. The work has been done with the aid of an expenses grant from the Medical Research Council. The cost of the expensive apparatus was defrayed by a grant from the Sir Halloy Stewart Trust of London.

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## ACUTE HÆMORRHAGIC ENCEPHALITIS ASSOCIATED WITH ACUTE RHEUMATISM

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(PLATES XLI AND XLII)

THIS case of acute hæmorrhagic encephalitis (for literature see Baker, 1935, Russell, 1937) is of interest because there was a clinical association with acute rheumatism but no evidence of specific rheumatic change in the brain. We know of only one other similar case (Alpers, 1928)

### *Clinical history*

S E, aged 8 years, was admitted on 2nd October 1937 to University College Hospital with the following history. Two days before admission she had complained of malaise, nausea and a pain in the left knee. The following day there was pain in the left wrist and left ankle and she felt cold. On the day of admission she had slight headache and a sore throat and the joint pains were more severe, especially in the left knee. There was never any symptom indicating involvement of the central nervous system, nor was there a history of vomiting.

She was one of a family of four healthy children. She had had diphtheria at 4 and measles at 6 years of age, otherwise the past history was uneventful. The father was alive, well and in work. The mother had died six years previously of cancer.

The patient was a well developed, rather than girl, lying very quietly in bed. She appeared to be only moderately ill and complained chiefly of pain in the left knee and to a less extent in the left ankle and left wrist. Temperature 100.5° F, pulse 125, respirations 30. She was flushed and perspiring slightly. The apex beat was forcible and best felt half an inch outside the nipple line. There was a soft systolic murmur at the apex, conducted into the axilla. The left knee was hot, swollen and acutely painful on movement of the joint or even on slight pressure. The left wrist and left ankle were both slightly tender and acutely painful on movement but not swollen. The right knee was slightly painful on movement. There were no abnormal physical signs in the lungs, abdomen or central nervous system. There were no nodules or skin rashes, nor were there any abnormal constituents in the urine.

The case was considered to be a straightforward one of acute articular rheumatism with carditis, and treatment with salicylates was started immediately. The patient was given 20 gr each of sodium salicylate and

sodium bicarbonate every four hours and was nursed on the principle of complete rest

By the third day after admission the temperature and the respiration rate had returned to normal and the pulse had dropped to between 90 and 100. The swelling and pain in the joints had disappeared, leaving only a slight residual stiffness of the left knee.

On the morning of the fourth day after admission the patient woke up and complained that there were "queer things" making a noise under her bed. It was at first thought that she had had a nightmare, but she persisted in this and other delusions, and towards the end of the morning was becoming restless, incoherent and unmanageable. At the same time there was a rise in the respiratory rate without any fever, by midday the respirations, 36 per minute, were deep and laboured. An examination at this time showed that there had been no change in the physical signs since admission except for the disappearance of the joint swelling and pain. A blood count taken on the previous day was returned at this time and showed hæmoglobin 55 per cent, R B C 2,800,000 and W B C 13,800, of which 86 per cent were polymorphs and 13 per cent lymphocytes.

A tentative diagnosis of salicylate intoxication was made, in spite of the moderate dosage accompanied with sodium bicarbonate which had been employed. She was given frequent glucose lemonade drinks, and hourly  $\frac{1}{2}$  dr doses of sodium bicarbonate were administered orally.

There was rapid deterioration in her condition. By 6 P.M. she was comatose, the temperature had risen to 104° F and the pulse to 120, while the respirations, 60 per minute, were deep and gasping. At this time a slight right external strabismus made its appearance for the first time and remained as the only positive finding in the central nervous system except for absent deep and superficial reflexes. Rectal glucose salines were administered and an intravenous drip was attempted but failed. The temperature and pulse rose and she lapsed into deeper coma, with persistence of the deep almost stertorous breathing. Just before death the temperature was 108° F, the pulse 160 and the respirations 60. She died at 3.30 A.M., six days after the onset of the initial symptoms.

### *Post-mortem examination.*

The post-mortem examination (210/37) was carried out 9½ hours after death by Professor G. R. Cameron.

The body was that of a fairly well nourished, well built girl, markedly pale, with a few purpuric spots on the back and neck and over both knee joints. No swelling of joints, no rheumatic nodules, no enlarged lymph glands.

*Brain*, 1230 g. Numerous small subarachnoid hæmorrhages, but no evidence of meningitis. On section (fig 1), well defined hæmorrhages were seen scattered throughout the white substance of cerebrum, cerebellum and pons and varying in diameter from 1 mm to 2 cm. The large hæmorrhages showed much fibrinous deposit in contact with the adjacent brain substance and this could be shelled out cleanly. No obvious aneurysms in the circle of Willis or within the brain. Ventricles not dilated, venous sinuses normal. Middle ears clear.

*Heart*, 100 g. Very little pericardial fluid. Petechiæ and small

F. CEPHALITIS IN RHEUMATISM



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*Heart*, 100 g. Very little pericardial fluid. Petechiæ and small



ENCEPHALITIS IN RHEUMATISM



FIG. 1.—Sagittal section through cerebellum, showing three large and numerous small hemorrhages in the white matter.



hæmorrhages in parietal and visceral pericardium at base. No obvious hypertrophy. Cavities dilated. One large atheromatous patch on aortic cusp of mitral valve, tiny warty vegetations on mitral valve, other valves clear. A little wrinkling of the endocardium of the left auricle. A few tiny atheromatous plaques around the orifices of the coronaries, rest of aorta and veins normal.

The other relevant findings were few. There was enlargement of both tonsils; these contained pockets of pus from which hæmolytic streptococci had been grown. Numerous petechiæ were present in the peritoneal coat of the transverse colon and in the omentum. The remaining organs presented no abnormality.

*Cultures* of blood and brain *post mortem* showed no hæmolytic streptococci.

### *Histology*

Microscopic examination of the brain shows numerous small and large ball and ring hæmorrhages confined to the subcortical white matter, internal capsule, pons and cerebellum. All stages in the development of hæmorrhage can be traced from vessels with a few red corpuscles in the walls and just outside the adventitia up to large macroscopic effusions of blood associated with much tearing of brain substance. Many vessels show intense congestion without hæmorrhage, others are filled with conglutination thrombi. The perivascular space is frequently distended with eosinophilic coagulum. Large hæmorrhages show central structureless clot thickly mottled with dark brown pigment, but the prussian blue reaction is negative. There is no evidence of inflammatory reaction nor commencing organisation around any of the hæmorrhages. The walls of a few medium-sized vessels contain polymorphonuclear leucocytes and mononuclear cells. Many of the latter contain brown pigment and tiny droplets of lipid staining yellow with Scharlach R.

No actual break in continuity of the walls of the smaller blood vessels could be determined despite careful search of 138 serial sections. Hæmorrhage appeared to occur in the early stages by diapedesis. Disorganisation of the vessel walls was seen only in association with the larger, older hæmorrhages.

Weigert-Pal sections show foci of perivascular demyelination (fig. 2) outlining accurately the ball and ring hæmorrhages or appearing as longitudinal sleeves. Commencing microglial reaction can be demonstrated occasionally. In no instance have these areas of demyelination been seen apart from hæmorrhage. Neither have non-hæmorrhagic perivascular areas of necrosis been found. There is no evidence of specific rheumatic lesions in the brain substance. The leptomeninges show no inflammatory reaction and the vessels at the base of the brain and the perforating vessels are free from change.

Sections of the left auricle, left ventricle and interventricular septum show Aschoff nodules (fig 3) in various stages of development, with much fibrinoid degeneration in the endocardium of the left auricle. Small vegetations are numerous on the mitral and tricuspid valves and are composed of platelets and fibrin with a diffuse round-celled infiltration beneath in the valve substance. Aschoff nodules are also present here. No organisms can be demonstrated by histological methods.

The remaining organs present no microscopic abnormality.

### *Summary*

This case is one of an 8-year-old girl admitted to hospital with moderately severe rheumatic fever with articular and cardiac manifestations. After responding satisfactorily to salicylate treatment she suddenly developed nervous symptoms and hyperpyrexia and became comatose in 12 hours. Death followed after 18 hours. *Post mortem*, the heart showed recent rheumatic infection of the muscle and valves, and the brain acute hæmorrhagic encephalitis.

We are indebted to Professor Cameron for much help in the investigation of this case and to Dr W. Pearson for allowing us to make use of the clinical notes.

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ENCEPHALITIS IN RHEUMATISM



FIG 2—

old myelination Weigert Pal  $\times 120$

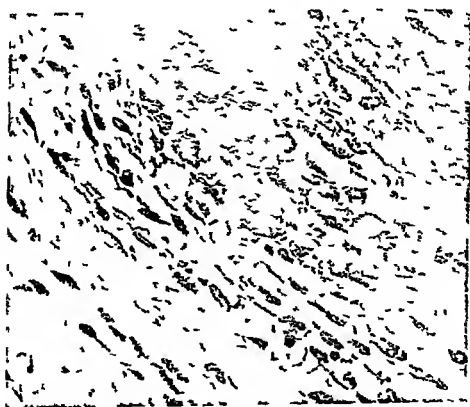


FIG 3—Aschoff nodule in myocardium H and E  $\times 100$



# LYMPHADENOID GOITRE A STUDY OF THIRTY-EIGHT CASES

DOROTHY M VAUA

*From the Pathology Unit Royal Free Hospital, London*

(PLATES XLIII—XLVI)

IN 1896 Riedel described two cases of iron hard goitre, one of which he had removed twelve years previously. Microscopically he found fibrous tissue and accumulations of round cells more or less destroying the gland. Sixteen years later Hashimoto (1912) published four cases of goitre presenting varying degrees of atrophy of the acinar epithelium, numerous lymph follicles and widespread new connective tissue and round cell infiltration. About 63 similar cases have now been reported.

Many recent writers, including Ewing (1928), consider that Riedel's struma and Hashimoto's struma lymphomatosa are different stages of the same pathological condition. Eisen (1934) describes 7 cases and after a full discussion concludes that they are different morphological manifestations of the same disease process. From a review of published cases Benson (1935) considers that there is an interlocking as regards symptoms, age incidence and pathology. Shaw and Smith (1925-26), who report 6 cases, come to the same conclusion, and a like opinion is held by Heyd (1929) and by Poer, Davison and Bishop (1938). On the other hand Graham and McCullagh (1931) consider Riedel's struma to be a different condition from the lesion described by Hashimoto on the ground that, though the supposedly later stage of the disease, it occurs in younger persons and is frequently a unilateral or localised process. This view is supported by Howard (1934), Gilchrist (1935), and Clute, Eckerson and Warren (1935).

A study of the histology of the 38 cases reviewed in this paper confirms the opinion that Riedel's struma and Hashimoto's struma are stages of the same pathological condition. Microscopically three different stages can be distinguished, early, intermediate and late, which merge into each other. The pathological changes are the same in kind in all three stages, the degree of change is the varying factor. The essence of the condition is the extent of the damage to the acini by increased lymphadenoid tissue and accompanying fibrosis.

## Early cases

Fifteen cases fall into the early stage. Here the chief features are scattered areas in which the acini are compressed and distorted by diffuse infiltration with lymphocytes and plasma cells and by

accompanying slight fibrosis, but for the most part, the acini are normal or show varying degrees of epithelial hyperplasia. Desquamated epithelial cells within the acini and giant cells formed from the acinar epithelium may be present. Colloid storage is usually somewhat reduced and the colloid may be vacuolated in the areas of epithelial hyperplasia. Throughout the gland large foci of lymphadenoid tissue are numerous, while fibrosis is slight (figs 1 and 2).

#### Intermediate cases.

Twelve of the 38 cases belong to the intermediate stage (figs 3-6) and show considerable compression and distortion of the acini by diffuse infiltration with lymphocytes and plasma cells and by fibrosis, but many areas of thyroid parenchyma have survived. Evidence of previous epithelial hyperplasia is usually well marked. Giant cells, apparently arising from the acinar epithelium as a result of compression, and intra-acinar plugs of desquamated cells may be present. Colloid storage is slight or moderate. Large foci of lymphadenoid tissue are numerous as well as diffuse cellular infiltration. A moderate degree of fibrosis is present, both diffuse and in broad bands.

#### Late cases.

Of the remaining eleven cases, nine appear to show identical changes (figs 7-10); in these the acinar structure of the thyroid is almost entirely destroyed by fibrosis and infiltration with lymphocytes and plasma cells. Scattered collections of compressed, distorted and atrophied acini and irregular groups of epithelial cells are all that remain of the thyroid parenchyma. Some evidence of previous epithelial hyperplasia can usually be found in some areas. Giant cells are commonly found, they appear to be derived from the acinar epithelium as a result of compression by the lymphocytic infiltration and fibrosis. Colloid is very scanty. Foci of lymphadenoid tissue are present and may be numerous, as well as diffuse infiltration with lymphocytes and plasma cells. Widespread diffuse fibrosis and broad bands of fibrous tissue stretch irregularly through the gland. In the remaining two late cases the appearance is atypical. One (fig 12) is from a woman of 40 who had had a goitre for nineteen years and no other symptoms, the other (fig 13) is from a man of 32 years who had noticed a swelling in the neck for three months and who had had some difficulty in swallowing; his basal metabolic rate before operation was  $-18.5$  per cent. In these two cases, in addition to the changes characteristic of the late stage of lymphadenoid goitre, an unusually large number of giant cells of the foreign body type, mostly arranged in tubercle-like follicles (figs 12 and 13), are present, they appear



LYMPHADENOID GOITRE

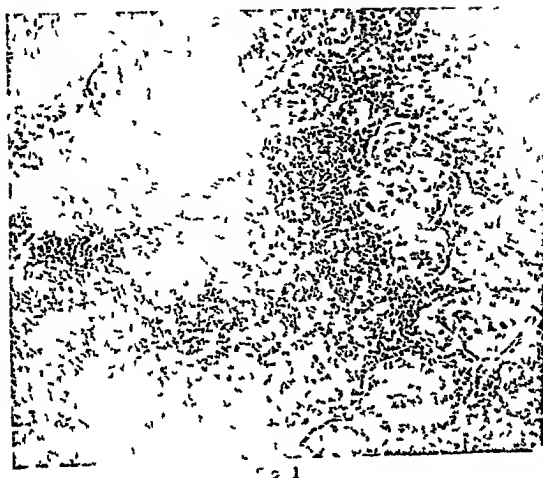


Fig 1



Fig 2

Early stage of lymphadenoid goitre  $\times 112$







to be derived from the acinar epithelium. Endarteritis is also seen (fig 14). Prolonged search for tubercle bacilli gave a negative result.

Macroscopically all these thyroids with one exception, are uniformly affected (average weight about 70 g, largest 225 g), being solid, firm or hard and of a pink fleshy appearance on section. Those which have the histological appearance of the late stage are harder than the others and show irregular fibrosis, whereas some in the early stage are not so uniformly firm and solid, but present, on the cut surface, solid areas scattered amongst colloid-containing thyroid tissue. Only one, an early case, is macroscopically a nodular goitre containing a few adenomata; the surrounding gland tissue shows the changes of lymphadenoid goitre.

#### *Clinical aspects*

The average age and sex distribution in each group are given in table I. The figures for age are not significantly different.

TABLE I  
*Average age and sex distribution in each group*

Stage	No. of cases*	Average age	Sex	
			M	F
Early	13	41	0	14†
Intermediate	12	46	1	11
Late	9	48	4	6†

\* In 4 cases included in the histological study (— early and 2 late) the age was unrecorded.  
† In one case in each of these groups the age was unrecorded.

In 35 cases in which clinical details are available the incidence of thyrotoxicosis and of hypothyroidism in each of the three stages has been analysed (table II).

TABLE II  
*Incidence of thyrotoxicosis and of hypothyroidism in 35 cases of lymphadenoid goitre*

Stage	No. of cases	Thyrotoxicosis		Hypothyroidism	
		No. of cases	Percentage	No. of cases	Percentage
Early	13	7	53.8	2	15.4
Intermediate	12	3	16.7	5	41.7
Late	10	0	0.0	7	70.0

These figures suggest that thyrotoxicosis, prevalent in the early stage of lymphadenoid goitre, gives place in the later stages to hypothyroidism. For both conditions, the differences in incidence between the early and late stages of the disease are statistically significant.

The youngest patient was a boy of 10 years, whose only symptom was a generalised enlargement of the thyroid noticed for  $2\frac{1}{2}$  years. This goitre has the features of the intermediate stage. The oldest case was a man of 74 years with a large goitre, 225 g, noticed for three months, increasing in size and producing pressure symptoms. There were no signs of either hypothyroidism or thyrotoxicosis. Histologically this goitre belongs to the late stage.

The basal metabolic rate was calculated in 22 of the cases, and the lowest figure obtained, -35 per cent, was in one of the intermediate group.

Four out of the 7 cases of the early group in which there was some evidence of thyrotoxicosis had a slightly raised basal metabolic rate before operation, in the remaining three it was not determined.

### *Ætiology*

Both Riedel and Hashimoto considered the lesion in their cases to be chronic inflammatory, though Hashimoto found no evidence of any infective or toxic agent. In Graham and McCullagh's opinion Riedel's struma is a local inflammation and struma lymphomatosa a manifestation of a constitutional disorder of unknown ætiology. Attempts have been made to relate the condition to infections in the mouth and upper respiratory tract (Eisen, Shaw and Smith; etc). Meeker (1925) describes a case with a persistent posterior branchial body and suggests that infection may reach the thyroid through it. Ewing considers the lesion to be a benign type of granuloma, Scott Williamson and Pearse (1929) regard it as "an expression of a disturbance of the lymphogenic function of the gland."

Boyden, Collier and Bugher (1935), after considering Warthin's views (1930) on the histological effect of administration of iodine in exophthalmic goitre, summarise their opinion as follows —

"There is strong evidence, from both the clinical and pathological standpoints, that the cases should be grouped under the category of the Graves' constitutional thyroid (Warthin), and that the pathological changes peculiar to the disease are largely the result of an over-response on the part of the thyroid to prolonged iodine ingestion in certain individuals showing this constitutional abnormality. Inflammation of a chronic non-specific nature may be an associated ætiological factor in some cases, accounting for the extreme sclerosis and production of adhesions to surrounding structures."

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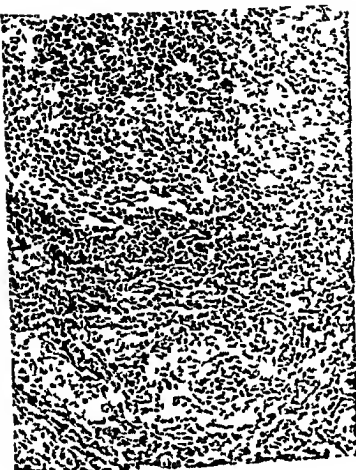


FIG 7

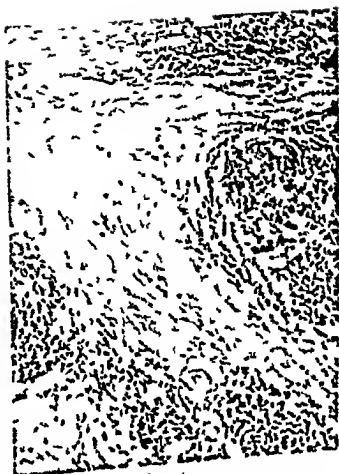


FIG 8

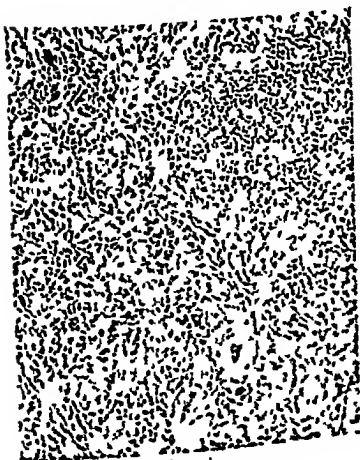
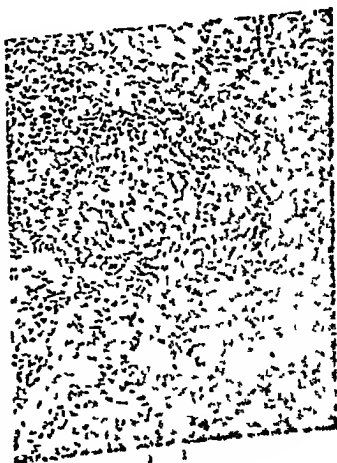


FIG 9



Intersecting the plane of the





D. G. H.



FIG. 11—Late stage of lymphoid nodules in thyroid.  $\times 112$



FIG. 12—Atypical late stage showing giant cells in tubercle-like follicles.  $\times 112$



FIG. 13—Atypical late stage showing giant cells in follicles.  $\times 112$



FIG. 14—Late stage showing a dense population of cells.  $\times 112$



From a study of the histology of these 38 cases, the most reasonable interpretation is that the condition is an involution of the thyroid. Tuberculosis, syphilis and infection with pyogenic organisms can be excluded. Since the average age incidence is 40 years, it might be thought that the involution is comparable with the changes in other glandular organs such as the breast, prostate and ovary, and that some disturbance of the balance of secretions of the ductless glands may initiate the condition. But the clinical evidence of thyrotoxicosis, especially in the early cases, correlated with the histological findings of decreasing degrees of epithelial hyperplasia and increasing destruction of the acini as the condition progresses, points to some excessive involution following a thyrotoxicosis of mild degree. In some of the cases, it may well be that a non-specific chronic inflammation is superimposed.

### Summary

1 The relationship between Hashimoto's struma lymphomatosa and Riedel's iron-hard goitre is discussed.

2 From a study of 38 cases it is concluded that these conditions are the earlier and later stages of the same pathological process.

3 Histologically the cases examined fall into three stages, early, intermediate and late, which agrees with the clinical findings.

4 The aetiology of the condition is discussed, and it is concluded that lymphadenoid goitre should probably be regarded as an excessive involution of the thyroid following mild thyrotoxicosis.

I wish to thank Mr H W S Wright F.R.C.S. and Dr R G Weller for permission to include in this series one each of their cases, for the remainder I am indebted to Mr C A Jell, F.R.C.S., and I thank him for permission to use the relevant clinical records.

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# THE TRANSMISSION OF THE ROUS FILTERABLE AGENT TO CHEMICALLY INDUCED TUMOURS

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(PLATE XLVII)

IN the Report of the British Empire Cancer Campaign for 1934 I referred to experiments in which it had proved impossible to show the presence of a filter-passing agent in cancerous growths of fowls induced by dibenzanthracene and tar. This result was not in agreement with that previously published by McIntosh (1933), who demonstrated the presence in tar-induced tumours of a filterable agent comparable to the well known agent of the Rous and other bird tumours. I pointed out that if chemically induced tumours in fowls contain a filterable active agent, it appeared to be exceptional and not the rule. In 1928 Sturm and Murphy had also failed to obtain any evidence of the presence of a filterable active agent in tar tumours of fowls. The suggestion has been made that the positive results obtained by McIntosh may have been due to the fact that some of the birds he used carried in their tissues an infective agent of the bird leukæmia type at the time they were bearing the tar tumours, and that it was this adventitious agent which really stimulated the formation of new tumours and not the specific product of the tar tumours. Even on the basis of this explanation the subject seemed of interest. I proceeded, therefore, to see whether the carcinogenic agent produced in a Rous sarcoma could pass to a chemically induced tumour in the same bird, and if so, whether the cancerous growths resulting from such a filterable agent would simulate the chemically induced or the Rous sarcoma as regards structure and metabolic activity. In the Report of the British Empire Cancer Campaign for 1935 I gave a résumé of the results obtained. It was then stated that in the case of birds bearing at the same time both dibenzanthracene and Rous tumours, cell-free filtrates made from the dibenzanthracene tumours and injected into fowls resulted in new tumours and that these, both structurally and according to metabolic tests, had the properties of a Rous sarcoma and not of the original dibenzanthracene tumour.

These experiments demonstrated that the filterable agent of a Rous sarcoma could pass readily into a chemically induced sarcoma which itself had no filterable agent so far as could be detected by the methods used

The present publication supplies the evidence upon which these statements were made. It suggests further that the presence of the Rous agent in the dibenzanthracene tumour does not alter the character of the latter, so that although cell-free filtrates induce Rous tumours, an injection of the cells of the infected dibenzanthracene tumour produces another dibenzanthracene tumour and not one of the Rous type

More recently some experiments have been made in which fowls carried three kinds of tumours, dibenzanthracene tumours in one breast, tar tumours in the other breast and Rous tumours in each leg. One of these experiments is reported and evidence is given of the passage of the Rous agent to the tar tumour

#### *Method of experiment.*

The method adopted was to inject an inoculum of cells of a chemically induced tumour, in most of the experiments of the dibenzanthracene type, into the breast muscles of fowls. At varying stages of development of these tumours cells of a Rous sarcoma were injected into both legs of each fowl. In the course of time the fowls had a dibenzanthracene tumour in one or both breasts and a Rous sarcoma in each leg. When the fowls were killed or died, cell-free filtrates of the dibenzanthracene tumours in the breasts were made and injected into normal fowls. In other fowls the cells of the dibenzanthracene tumours were injected. From any subsequent tumours which developed, injections of cells and cell-free filtrates were made for a varying number of generations in different experiments.

Throughout the work here reported, the preparation of the cell inocula and cell-free filtrates and the amounts injected have been kept approximately constant with two exceptions. For the filtrates, about 1 part of tumour tissue was ground up with silver sand and extracted with 9 parts of normal saline made with tap water. This extract was centrifuged and then filtered through paper pulp, 1 c.c. doses of the filtrate being inoculated into each breast of the fowls. When cells were used 0.05 c.c. of scissors-minced tissue was used for inoculation into each breast. In one of the early experiments (1934) recorded below (exp. A), 2 c.c. of the cell-free filtrate were injected. It might have been better to have injected this amount in the other experiments instead of 1 c.c., since the positive results in this case were two out of three, whereas usually the proportion of successes was less.

The methods whereby differentiation of the two types of tumours—dibenzanthracene and Rous—was made will now be mentioned.

(1) In my experience, a cell-free filtrate of an ordinary dibenzanthracene or tar tumour of a fowl does not produce another tumour on inoculation as does that of a Rous tumour.

(2) The appearances of a dibenzanthracene or tar sarcoma and a Rous sarcoma, macroscopically and microscopically, are usually different, the first two are more cellular and solid, the Rous tumour is mucilaginous and of loose texture. The diagnosis of these types of tumours by their histological appearance is illustrated in figs. 1-4 (pl. XLVII). A fowl carried a dibenz-

anthracene tumour in the breast and a Rous tumour in each leg. The structure of the former is seen in fig 1, of the latter in fig 2. Cells of the dibenzanthracene tumour were inoculated into another fowl and gave rise to a tumour of the same type (fig 3). On the other hand, a cell free filtrate of the same dibenzanthracene tumour (fig 1) gave rise to a tumour of typically Rous appearance (fig 4).

Occasionally, however, a Rous tumour of the slow growing type is found which is more cellular and more difficult to differentiate from the chemically induced tumours.

(3) It was hoped that a comparison of the metabolic properties of the tumours might assist in distinguishing them, but it is now doubtful whether this method is any better than the histological examination, although of course it provides additional support for diagnostic purposes.

It is true that the metabolic characters of these two tumours are sufficiently different to allow classification when typical tumour tissue of each type is examined. Examined by the Warburg method, with the Dickens-Suncer apparatus (1930, 1931), the greatest and most constant metabolic difference is the glycolytic power of each estimated in terms of the dried weight. For the Rous sarcoma of typical structure the glycolytic action  $Q_{O_2}^g$  per mg dried tissue per hour varies between 7 and 9. The corresponding figure for the fowl dibenzanthracene tumour tissue is 5.6. The other two figures given by the Warburg technique— $Q_{CO_2}$ , the carbon dioxide produced by oxidation per mg dried weight per hour and  $Q_{O_2}$ , the oxygen uptake per mg dried weight per hour—vary widely in different tumours of the same type, and the range is too similar to provide a measure of differentiation, although the more typical and rapidly growing the Rous tumour the greater is the oxygen uptake as compared with that of the dibenzanthracene tumour.

It is of interest to note that the percentage of dried substance in Rous tumour is usually much smaller than that of the fowl dibenzanthracene tumour—an average of 10.11 per cent as compared with the 18 per cent in the case of the dibenzanthracene tumour tissue. This figure, obtained merely by drying tissue slices of the respective tumours, is in itself a good indication of the type of tumour, but may fail in differentiating the uncommon, hard, slow growing Rous tumour from the typical dibenzanthracene tumour. These various methods, the mode of propagation of the tumours, the gross and histological appearance, the relative metabolic activity and the percentage dried weight, have been used to differentiate the types of tumour produced from the experimental cell and cell free filtrate inoculations.

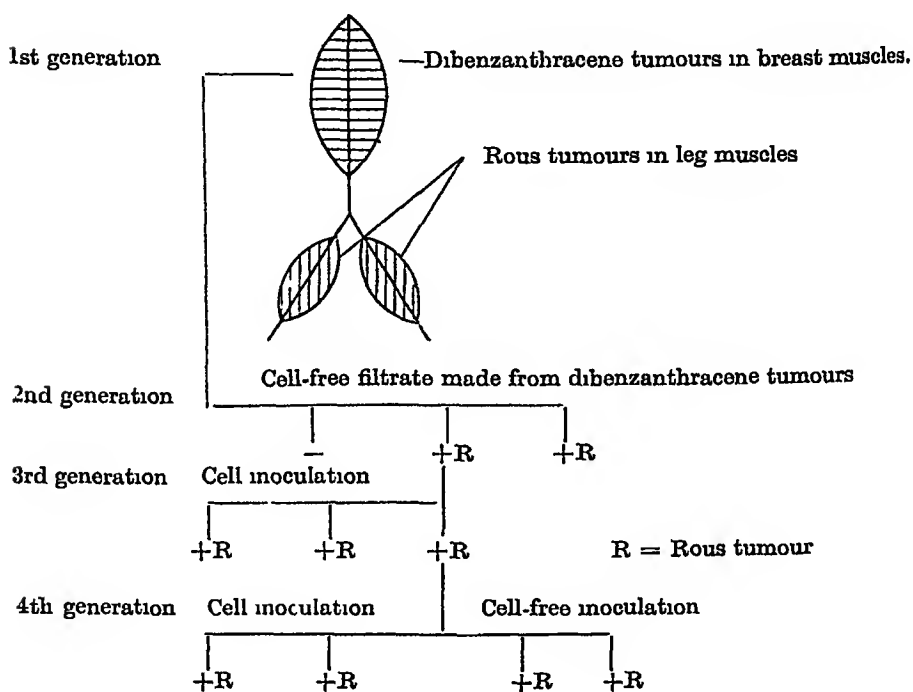
## EXPERIMENTAL RESULTS

The following may be taken as an example of one of the early experiments—

**Experiment A** A fowl was inoculated into both breast muscles with dibenzanthracene tumour tissue (FD/2). 11 days later there were two palpable tumours, after another 13 days, when the tumours were large, both legs were inoculated with Rous sarcoma cells. All four tumours were growing when the fowl died, 38 days after the beginning of the experiment. A paper-pulp filtrate (cell free) was made of the breast (dibenzanthracene) tumours; thus, on inoculation into three fowls, produced tumours in two of them. These tumours

were further propagated both by cell and cell-free inoculation, resulting in each case in typical Rous tumours

It is of interest to note that the dibenzanthracene tumour (FD/2) grew much more slowly at the time of this experiment in 1934 than in later years. This explains why there was a longer interval after inoculation between the dibenzanthracene and the Rous products in this than in later experiments



EXPERIMENT A

Here, then, is an instance of a dibenzanthracene tumour containing Rous filterable agent, transmitted to it from Rous sarcomata in the leg, which was propagated, like Rous tumours, on the injection of a cell-free filtrate. In this instance the cells of the original dibenzanthracene tumour were not inoculated. In that case, from analogy with many other experiments (see expts B and C), a dibenzanthracene tumour and not a Rous tumour would have grown. Figs 1-4 illustrate such an experiment.

**Experiment B** The following experiment also shows that the original dibenzanthracene tumour retains its own properties on cell propagation, even when it contains sufficient Rous filterable agent to give rise to a tumour of the Rous type.

A fowl was inoculated, into the right breast only, with dibenzanthracene tumour cells (FD/2). Seven days later, Rous tissue was injected into both legs. After a further period of 11 days the fowl, now bearing fairly big tumours in the right breast and both legs,



PRODUCTION OF TUMOURS IN A TYPICAL EXPERIMENT

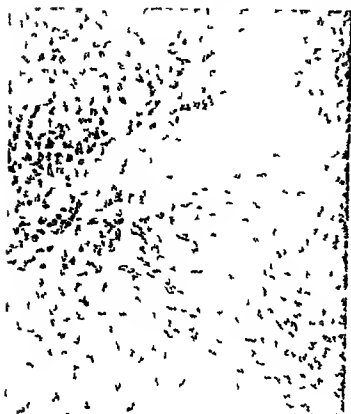


FIG 1—Typical

The c

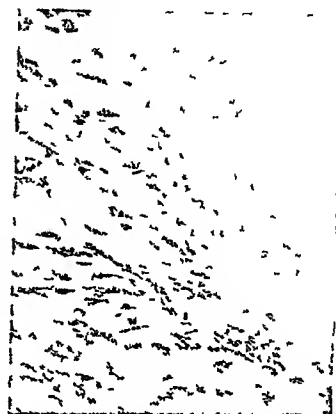


FIG 2—Typical Rous tumour of a fowl  $\times 350$

... and leg respectively of the same fowl

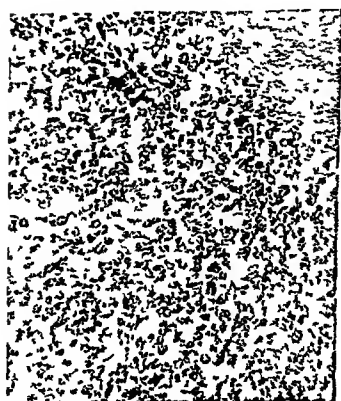


FIG 3—Dibenzanthracene tumour in a fowl produced by a cell inoculum from the tumour represented in fig 1  $\times 350$

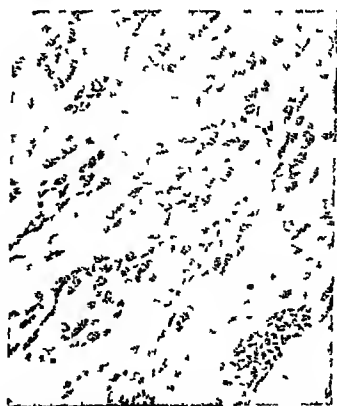
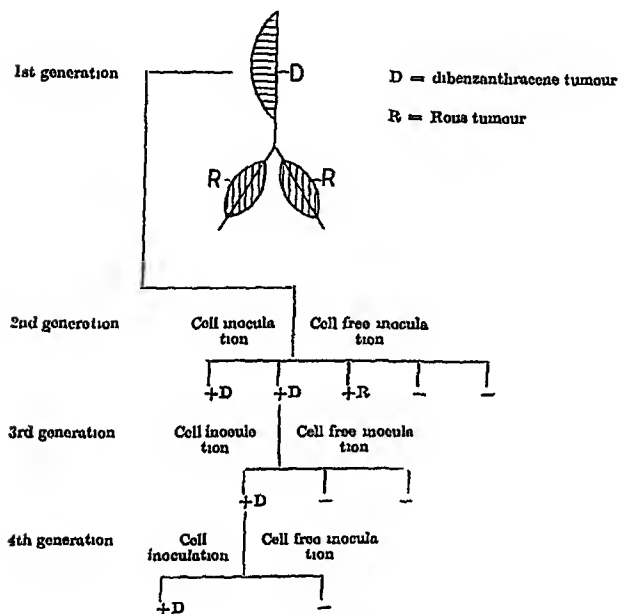


FIG 4—Rous tumour in a fowl produced by a cell free inoculum made from the dibenzanthracene tumour represented in fig 1  $\times 350$



was killed Cells and cell-free filtrate from the breast tumour were inoculated into other fowls as below



EXPERIMENT B

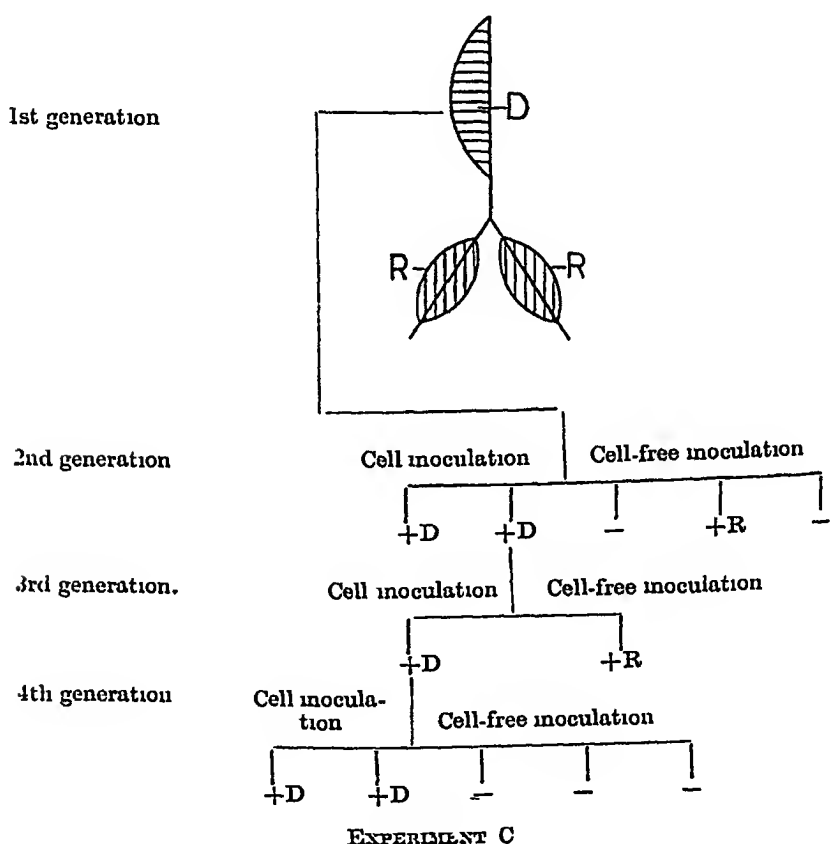
In this case a typical Rous tumour was produced, in 1 bird out of 3, by inoculation with a cell-free filtrate of the original dibenzanthracene tumour, the cells themselves, however, produced dibenzanthracene tumours in 2 birds injected

It is usually only possible to get evidence of the presence of the Rous filterable agent in the dibenzanthracene tumour when a Rous tumour is growing in the same bird. In two experiments, however, there was evidence of the presence of the Rous agent in the second generation of both dibenzanthracene and tar tumours, i.e. tumours which had grown in birds which themselves had no accompanying Rous tumour. This is illustrated in the next experiments

*Production of Rous tumours from the second generation of chemically induced tumours by cell-free filtrates*

In the first experiment the tumour from which the cell-free filtrate produced a Rous sarcoma was of the dibenzanthracene type.

**Experiment C.** A fowl was inoculated into the right breast muscle with tissue from a dibenzanthracene tumour (FD/2). Eight days later Rous tissue was inoculated into both legs. The fowl was killed 13 days after the second inoculation, when it had tumours in the right breast and in both legs. Cells and cell-free filtrates of the dibenzanthracene tumour were then inoculated into other fowls, and the experiment proceeded as follows



It will be seen in this experiment that, in addition to the Rous tumour produced by a cell-free filtrate of the original dibenzanthracene tumour, another Rous tumour was produced by a cell-free inoculation of a second generation dibenzanthracene tumour, although this tumour had never itself been in association with a Rous sarcoma.

In the following experiment an active cell-free filtrate was obtained from the second generation of a *tar* tumour. No active

filtrate was obtained from either the first or second generation of the *dibenzanthracene* tumour in the right breast of the same fowl

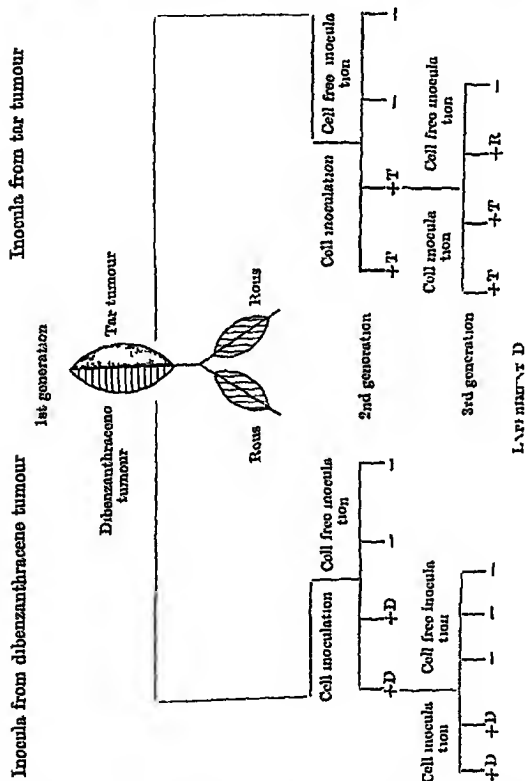
**Experiment D** A fowl was inoculated on the same date as follows

Right breast—0.05 c.c. *dibenzanthracene* tumour tissue

Left „ —0.05 c.c. tar tumour tissue

Both legs (intramuscularly)—0.05 c.c. Rous tumour tissue

Eighteen days later, when the fowl was killed, there were large



tumours in both breasts and fairly big tumours in both legs  
There were no metastases Cell suspensions and cell free filtrates

were made from both the dibenzanthracene and tar tumours and injected into other fowls

It will be seen (p 453) that, whereas the cell-free filtrate of the first generation of the tar tumour gave negative results on inoculation, that of the second generation produced a Rous sarcoma

In this experiment the following points may be noted

- 1 The original bird carried three types of tumours, Rous in the legs, a dibenzanthracene tumour in the right breast and a tar tumour in the left breast

- 2 Inocula of cells and cell-free filtrates from both the tar tumour and the dibenzanthracene tumour were injected into other fowls

- 3 Injection of a cell-free filtrate of the original tar tumour was negative whereas an injection of a cell-free filtrate made from the second generation of tar tumour produced a Rous sarcoma

- 4 A cell-free filtrate of the dibenzanthracene tumour from both first and second generations was negative

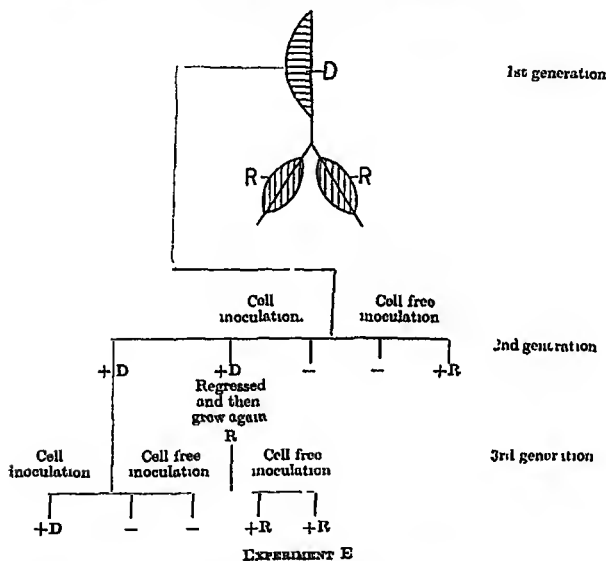
Of the many experiments carried out, expts C and D are the only two in which it has been possible to show the presence of the Rous agent in the second generation of chemically induced tumours by direct experiment.

#### *Regression of dibenzanthracene tumour and replacement by Rous sarcoma*

On two occasions an occurrence worthy of mention has happened. Dibenzanthracene tumours of the second generation, *i.e.* tumours grown from the cells of dibenzanthracene tumours of fowls also carrying Rous tumours, after showing good growth, regressed and after regression (in one case complete regression) other tumours grew in their place. The second tumours were typical Rous sarcomata in appearance and were readily propagated by cell-free filtrates. The following experiment illustrates this point.

**Experiment E.** A fowl was inoculated into the right breast muscle with dibenzanthracene tumour tissue (FD/2). After seven days it was inoculated into both legs with Rous cells and 14 days later it was killed, when it had fairly big tumours in the right breast and in both legs. Cells and cell-free filtrates of the dibenzanthracene tumour in the breast were then inoculated into other fowls and produced tumours. The tumour grown from the cell-free filtrate was typically Rous in appearance. Of the two birds inoculated with cells from the original dibenzanthracene tumour, one was of typically dibenzanthracene structure and could only be propagated by cells, not by cell-free filtrates. The second developed

tumours in both breasts, which regressed, that on the left side completely. The tumour in the right breast did not completely disappear but started to grow again. At death, this right breast tumour was of the Rous type and was propagated easily by cell free filtrates. The following diagram illustrates these points



The changes in the breast dibenzanthracene tumour and its replacement by a Rous tumour can be seen in the following table

		4 days after inoculation	12 days	20 days	31 days	34 days	41 days
		Neg	Dibenzanthracene tumour				Rous tumour
			+	+	+	?	
Inoculation with cells of dibenzanthracene tumour carrying Rous virus	Right breast	Neg	+	+	+	?	++
	Left breast	Neg	+	+	+	?	Neg

In another experiment where the same kind of change occurred, both dibenzanthracene tumours in the breast muscles completely

disappeared, as far as palpation could indicate this, and other tumours grew which proved to be Rous sarcomata and could easily be propagated by cell-free filtrates

It is important to note that in both of these experiments the original dibenzanthracene tumours in the breasts regressed, in one case completely, before new Rous tumours grew at the same site. This indicates that the growth of the Rous cells did not kill the dibenzanthracene cells, but it does not exclude the possibility that the Rous agent killed the dibenzanthracene tumour cells. This, however, seems unlikely, for dibenzanthracene tumours in fowls occasionally regress in any case, and there did not appear to be any increase in the incidence of regression of chemically induced breast tumours when associated with Rous tumours in the legs of the same bird as compared with those growing alone.

An attempt was made to obtain direct evidence on this point by injecting large quantities of Rous agent either into dibenzanthracene tumours or into the blood stream of fowls bearing these tumours. The Rous filterable agent did not make the dibenzanthracene tumours regress, but in some of the birds in which it was injected directly into the dibenzanthracene tumour, Rous tumour tissue was produced and the final tumours seemed to be a mixed growth of Rous and dibenzanthracene tissue.

On the whole, therefore, it appears that in the experiments described above in which dibenzanthracene tumours regressed and were replaced by Rous tumours, the regression was not due to the presence in them of Rous virus, but that the process of regression liberated the Rous virus from the cells and concentrated it sufficiently to allow the transformation of connective tissue cells to Rous sarcoma cells.

It seems possible that the transmission of Rous virus to the second generation of chemically induced tumours may be more common than the direct experimental evidence indicates; for, as mentioned above, only in two instances has it been possible by direct inoculation to obtain evidence of the presence of Rous agent in the second generation of dibenzanthracene and tar tumours. Had larger quantities of cell-free filtrate been used in these experiments more positive results might have been obtained, but it has been the aim throughout this work to use the same technique and to inject approximately the same quantity of cell inoculum and of cell-free filtrate in all experiments (see p. 448).

#### DISCUSSION.

It has been shown that where a fowl carries both chemically induced and Rous tumours at the same time, the filterable agent of the latter is transmitted to and taken up by the former, so that a cell-free filtrate of a dibenzanthracene or tar tumour may give





ordinary Rous sarcomas. Normally, by the routine methods adopted in this laboratory, a cell-free filtrate of a Rous growth produces a palpable tumour in about 14 days. These facts suggest that the Rous filterable agent transmitted from a Rous sarcoma to a dibenzanthracene tumour is attenuated, and that its power to multiply in its new habitat is limited.

On the other hand it seems probable that the amount of Rous agent demonstrable in the second generation of dibenzanthracene and tar tumours can only be accounted for by its actual reproduction in this tumour. If this be the case, evidence of its presence in the third and later generations of dibenzanthracene tumours might be expected, but no such evidence has been obtained. When a Rous tumour begins to grow and replaces the regressed dibenzanthracene tumour, there is abundant production of the Rous agent. But the point at issue is whether the Rous agent multiplies in the dibenzanthracene tumour independently of Rous cells. It may be true that when the Rous agent is present in the second generation of chemically induced tumours, some Rous tissue is present and the tumour is a mixed one. Whether this is the case cannot be stated with certainty, but if this is the explanation, the amount of Rous tissue must be small. The question, therefore, whether the Rous agent increases in amount while present in the dibenzanthracene or tar tumour independently of Rous cell formation cannot be answered on the basis of present knowledge.

In a later paper, evidence will be given to show that most if not all of the normal organs of a fowl bearing a Rous sarcoma become invaded by the Rous agent in varying degrees, but are unaffected by it. The same fact seems to hold as regards the dibenzanthracene and tar tumours. They also become a receptacle for the Rous filterable agent but are apparently not otherwise affected by it. There is, however, as will be seen later, one big difference between a normal, chemically induced tumour of a fowl and the spleen or liver of a fowl carrying the Rous agent. Injection of liver or spleen cells containing Rous virus into another fowl produces a Rous sarcoma, whereas injection of the dibenzanthracene or tar tumour cells, although containing Rous virus, will produce a dibenzanthracene or tar tumour. Cell-free filtrates of the same liver, or spleen, or of dibenzanthracene or tar tumours will all produce Rous sarcomata.

These experiments show that a chemically induced tumour can be infected by a cancer virus agent and to that extent support the view that McIntosh's results may well have been due to such an infection in his fowls carrying tar tumours. The results in

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\* Reference was made to these experiments in the 12th and 13th Annual Reports of the British Empire Cancer Campaign (1935 and 1936)



4. In two out of many experiments, *cell-free filtrates* made from the second generation of chemically induced tumours—i.e. those which themselves had no association with a Rous sarcoma in the same fowl—produced a Rous sarcoma (3rd generation) which could be further propagated by cell-free filtrate. *Cells* of these chemically induced tumours, however, produced other (3rd generation) tumours of the same type as those originally chemically induced and these gave no evidence of containing the Rous agent

5. In two experiments, second generation dibenzanthracene tumours of fowls regressed and new tumours grew in their place. These new tumours were Rous in character and could be readily propagated by cell-free filtrates. There is no good reason to believe that the presence of the Rous agent actually made the dibenzanthracene tumours regress, as such regression may happen without the presence of Rous sarcoma in the same bird. Injection of Rous filterable agent into a dibenzanthracene tumour will not make it regress, but may produce Rous sarcomatous tissue in it. It appears, however, that when regression took place, the Rous agent exerted its effect and produced a second Rous tumour replacing the dibenzanthracene tumour.

Most of the expenses of this research were borne by the Yorkshire Council of the British Empire Cancer Campaign, to whom my thanks are due. Other expenses were paid by the Medical Research Council.

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inclusions in the fibroma cells similar to those seen in the myxoma cells. By adding heat-inactivated myxoma virus to a filtrate of fibroma virus Berry and Dedrick (1936) could transform the fibroma virus to that of the myxoma, with its clinical course and fatal effect on inoculated rabbits. Ledingham (1937) demonstrated elementary bodies by means of high-speed centrifugation of suspensions of fibroma and studied by means of agglutination tests the serological relationship of the myxomatosis and the fibroma. By filtration through graded collodion membranes and by centrifugation Schlesinger and Andrews (1937) estimated the diameter of the fibroma virus to be 125-175 m $\mu$ , that is about the same as that of vaccinia.

The histology of the filterable fibroma has attracted little attention by comparison with its immunological and serological characters. The following account deals especially with the earlier stages of the condition, the anatomical character of the fibroma, the occurrence of cytoplasmic inclusions in the fibroma cells and changes in the overlying epithelium.

#### *Methods and material.*

The investigation was carried out with the OA strain (Andrews and Slope), but in a few cases the IA strain was used. The ensuing account is based on the examination of 35 rabbits killed at different stages of the disease. Virus was introduced in the form of 5 per cent suspensions of emulsified testis material, fresh or kept for a short time in 50 per cent. glycerol. For intravenous inoculation suspensions were filtered through paper-sand-paper filter and collodion membranes (average pore diameter 1.38  $\mu$ ).

Tissues used for histological studies were fixed in Susa, sometimes in formol, and stained with hematoxylin and eosin, hematoxylin and van Gieson and by the Heidenhain-Mallory method. In addition the following staining methods were used: Weigert's fibrin stain, Mallory's phosphotungstic acid-hematoxylin, Heidenhain's iron hematoxylin, Mayer-Southgate nuclear carmine, Mann's stain, Scharlach R and Giemsa. Sinclear preparations were stained with Giemsa or anilin blue.

#### *Clinical picture*

After *intracutaneous* inoculation of 1:100 or stronger suspensions of virulent testis material a colourless or slightly pinkish papule is just visible on the 3rd to the 5th day, with dilutions of 1:1000-1:10,000 the first visible lesion appears on the 5th to the 8th day. The edges of the papules, at first not always well defined become marked and sharp after a few days. The nodules grow progressively in all directions and appear after a week as elevated, circumscribed tumours with wall-like edges. Sometimes the first reaction appears as a group of small nodules which soon become confluent. The nodules are always freely movable over the underlying subcutaneous tissue. The size of the tumour depends on the amount and strength of virus injected. After the injection of 0.1 c.c. of a 5 per cent. testis suspension the maximum size of a tumour is on the average 2.5 x 2.5 cm. and 1-2 cm. in thickness. Small tumours are most likely to develop

in small or poorly nourished animals. The bigger lesions often spread downwards in the direction aided by gravity. At first the tumours have a rounded shape, at a later stage they are flatter. Ten to eighteen days after the inoculation of virus the reaction has usually attained its maximum and after a short stationary period the tumour rapidly regresses.

The overlying skin shows at first slight reddening and is tense and glistening especially over the top of the tumour. Soon—sometimes after only a few days—the epithelium over the tumour shows a yellowish discolouration and slight dryness. Often this is most pronounced at the periphery in the form of a thin yellow ring surrounding a central paler and duller area. Almost regularly there appear small superficial hæmorrhages over the top of the tumour. These changes often appear at the same time and to the same extent over small (virus dilution 1:1000) and over larger tumours. If they appear early they will, during the later progress of the tumour, occupy its central part, while the periphery of the nodule is covered by normal epithelium. The superficial changes gradually increase and assume in most cases a pronounced hæmorrhagic character, larger tumours often show a central umbilicated hæmorrhagic scab surrounded by raised walls.

A central deepening of the surface is often the first sign of incipient regression, indicating early necrosis of the inner parts of the tumour. In addition the margin of the hæmorrhagic scab shows suppuration and almost regularly there appears during regression a demarcating inflammation of purulent character between the tumour and the surrounding tissue. The tumour is consequently attacked from two directions, partly from the periphery, partly through necrosis of its inner parts. The first signs of regression appear as a rule 14-20 days after the inoculation of virus and after a further 7-14 days the tumour has completely disappeared, leaving only a scab loosely attached to the skin. Small tumours tend to disappear earlier than larger ones. Only on very rare occasions does the skin tumour persist for a longer time, we have once seen a large skin fibroma, which only disappeared after two months. Tumours covered by intact epithelium usually persist longer than those with necrotic changes in the overlying skin.

The cut surface of the tumour is white, moist or shiny and slightly bulging, with a hard rubbery consistency. The central part is often looser and moister than the periphery. During regression the tumour tissue often assumes a soft consistency. At the time of complete regression the surface is transformed into a thick, half liquid, surface of the underlying muscles and occurs.

On *epidermal* inoculation after scarification Shope obtained only inconstant results in the form of slight reddening and thickening of the skin of short duration. In most cases, however, probably through using a stronger virus, we obtained more definite results. The skin was scarified over a rather large area and the virus suspension heavily rubbed in with a glass rod until the skin was almost dry. On the 4th day after inoculation the skin showed slight reddening and cedematous thickening and in a few days the scarified area was transformed into an elevated, intensely red fibromatous "pancake," covered by thin, glistening epithelium, often the skin was turned into thick, stiff folds. Soon there appeared small hæmorrhages or yellowish patches in the superficial layers. The regression of the lesion occurred through a shrinkage of the surface, which often became gradually covered by a thick scab, and through a purulent infection inside the tumour or at the edge of the surrounding tissue.

Shope (1932a) has given an exhaustive account of *subcutaneous* and *intratesticular* inoculation of virus. After *intramuscular* injection the reaction appears first as a rather diffuse, whitish infiltration in the muscle but assumes later a circumscribed, hard, tumour-like character. The fibroma may be attached to the intramuscular septa but is often embedded in the muscle substance. As in the skin, the consistency of the tumour during regression gradually becomes softer and the colour more yellowish, especially at the border. The tumour produced after inoculation by any of these routes usually persists for a longer time than after intracutaneous injection.

*Intraperitoneal* inoculation of virus is in most cases without effect but in one rabbit I obtained positive results. After intraperitoneal injection of 10 c.c. of 5 per cent testis suspension the animal gradually became emaciated, with loss of appetite and weight, and died one month after the inoculation. In addition to a rather big nodule in the abdominal wall corresponding to the needle track the rabbit showed at autopsy an annular fibromatous tumour in the cæcum close to the orifice of the ileum and obstructing the intestinal lumen. In the mesentery close to the cæcal tumour was a hard irregular fibromatous mass. The cæcum was filled with thick material, the rest of the intestine being thin and collapsed. In addition to these changes, which indicate a deposition in the intestinal wall of virus at the site of injection, the under surface of the diaphragm showed dissemination of small fibroma nodules, some of them confluent and forming flat plaques. They were white and of a hard consistency. The positive result in this case was perhaps facilitated by an infectious irritation of the peritoneum from the damaged bowel.

*Intravenous* inoculation of virus is, in our experience as in that





At this stage the tumour often has an inflammatory, granulomatous appearance, due to the extensive cedematous exudation and abundant inflammatory cells inside and around it. The hard consistency of the tumour is apparently caused by the edema and not by the presence of fibrils. A histological resemblance to myxomatosis is obvious in many cases (fig 4).

At a later stage, however, the tumours often assume a more neoplastic character microscopically (fig 5). The intercellular edema is less pronounced and the picture is more cellular. The cytoplasm of the fibroma cells is increased and the cells are not so loosely arranged. The nucleus is more irregular and the chromatin dispersed into a number of larger, deeply staining blocks. A fine mesh of intercellular fibrils gradually develops, chiefly visible at first in silver-impregnated or Mallory-stained specimens but later assuming a collagenous appearance. The inflammatory cells inside the tumour tissue gradually disappear and are now chiefly localised at the periphery. The tumour in this way gradually comes to consist of one type of cell, *i.e.* fibroma cells, and the varied cell composition of the first granulomatous stage is no longer seen. This transformation varies however in its extent from case to case, sometimes it appears only in some areas of the tumour, sometimes the condition maintains its inflammatory character until regression and only in cases where the tumour persists for a sufficiently long time will the above described more tumour-like structure appear. Owing to the degree of cedematous exudation skin tumours retain in many cases a fibromyxomatous appearance.

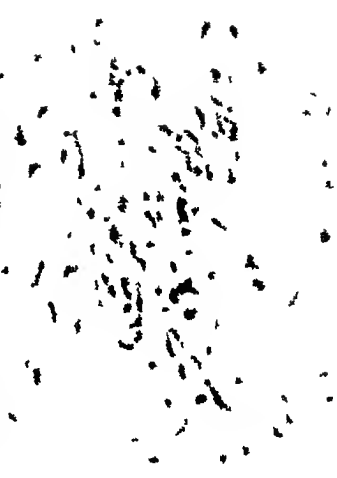
The inflammatory reaction in the surrounding tissue is usually most pronounced in the subepithelial layer and is connected with changes in the overlying epidermis. Owing to mechanical factors, especially the pressure of the accumulating edema in the underlying tissue, the epithelium over the tumour regularly shows more or less pronounced degenerative changes in the form of an cedematous loosening of the cells, necrosis and vesicle formation. The inflammatory reaction in the underlying tissue is gradually increased, the thin capillaries show congestion, and small hæmorrhages in the superficial layers of the tumour make their appearance. The superficial necrosis in this way gradually assumes a hæmorrhagic character. In addition to these rather non-specific lesions, however, the epithelium in some cases shows a peculiar type of reaction, occurring especially in the downward projections, where it comes in close contact with the fibromatous tissue (fig 6). Here the epithelium sometimes shows a hyperplasia with enlarged cells and in the cytoplasm of these cells appears a granular eosinophilic deposit. In addition many cells show vacuolation of the cytoplasm and degenerative changes in the nucleus while some have undergone



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The inflammatory reaction in the surrounding tissue is usually most pronounced in the subepithelial layer and is connected with changes in the overlying epidermis. Owing to mechanical factors, especially the pressure of the accumulating oedema in the underlying tissue, the epithelium over the tumour regularly shows more or less pronounced degenerative changes in the form of an oedematous loosening of the cells, necrosis and vesicle formation. The inflammatory reaction in the underlying tissue is gradually increased, the thin capillaries show congestion, and small haemorrhages in the superficial layers of the tumour make their appearance. The superficial necrosis in this way gradually assumes a haemorrhagic character. In addition to these rather non-specific lesions, however, the epithelium in some cases shows a peculiar type of reaction, occurring especially in the downward projections, where it comes in close contact with the fibromatous tissue (fig. 6). Here the epithelium sometimes shows a hyperplasia with enlarged cells and in the cytoplasm of these cells appears a granular eosinophilic deposit. In addition many cells show vacuolation of the cytoplasm and degenerative changes in the nucleus while some have undergone



At this stage the tumour often has an inflammatory, granulomatous appearance, due to the extensive oedematous exudation and abundant inflammatory cells inside and around it. The hard consistency of the tumour is apparently caused by the oedema and not by the presence of fibrils. A histological resemblance to myxomatosis is obvious in many cases (fig. 4).

At a later stage, however the tumours often assume a more neoplastic character microscopically (fig. 5). The intercellular oedema is less pronounced and the picture is more cellular. The cytoplasm of the fibroma cells is increased and the cells are not so loosely arranged. The nucleus is more irregular and the chromatin dispersed into a number of larger, deeply staining blocks. A fine mesh of intercellular fibrils gradually develops, chiefly visible at first in silver-impregnated or Mallory-stained specimens but later assuming a collagenous appearance. The inflammatory cells inside the tumour tissue gradually disappear and are now chiefly localised at the periphery. The tumour in this way gradually comes to consist of one type of cell, i.e. fibroma cells and the varied cell composition of the first granulomatous stage is no longer seen. This transformation varies however in its extent from case to case; sometimes it appears only in some areas of the tumour, sometimes the condition maintains its inflammatory character until regression and only in cases where the tumour persists for a sufficiently long time will the above described more tumour-like structure appear. Owing to the degree of oedematous exudation skin tumours retain in many cases a fibromyxomatous appearance.

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complete necrosis. The changes are of some interest in that similar but much more pronounced epithelial reactions are visible in the fibroma lesions of the cottontail rabbits.

The first histological sign of regression of the tumour is usually central necrosis. The tissue here stains more faintly and the fibroma cells gradually undergo necrosis without any characteristic preceding changes. Along with these internal alterations inflammatory changes around the tumour gradually increase and the neoplastic tissue is invaded from the periphery and especially along the vessels by inflammatory cells, chiefly leucocytes. Later, necrotising and inflammatory changes are intermingled, sometimes the semi-necrotic tumour is thrown off by means of a demarcating purulent inflammation, leaving a granulating ulcer, in other cases the tumour is gradually absorbed. Necrosis of the tumour regularly assumes in its superficial layers and sometimes also more deeply a hæmorrhagic character. It is often possible to find a complete necrosis of the fibroma without any infiltration of inflammatory cells into the tumour tissue, indicating that the former process is primary and the most important process in the regression of the tumour.

After inoculation of virus by *scarification* the histological changes appear in the same way as above described, the only difference being that the changes are localised to the superficial, subepithelial layers of the skin. The distended epithelium shows oedema and vesiculation and in many cells a granular eosinophilic deposit is visible. The infiltration of inflammatory cells into the fibroma tissue is usually rather pronounced.

The histological structure of tumours produced after *subcutaneous* or *intratesticular* inoculation is mainly as above described for the cutaneous tumour. Here also it is possible to distinguish a primary inflammatory stage, where the picture, besides a proliferation of fibroma cells, shows pronounced exudative changes in the form of oedema and scattered inflammatory cells inside the tumour tissue. Here also the origin of the fibroma cells in the earlier stages can be traced not only to the undifferentiated mesodermal elements between the collagen bundles, but especially to the surroundings of the capillaries, which are distributed in groups in the oedematous tissue and obviously serve as centres for the proliferation of the growing tumour. Shope describes how masses of young connective tissue cells can be seen radiating from blood vessels and forming nodules about them. The capillaries are formed of swollen endothelium of much the same appearance as the fibroma cells, but no outspoken endothelial proliferation can be seen. Owing to the fact that these tumours usually persist for a longer time than the cutaneous, they often assume a "riper" character. The arrangement of the fibroma cells is more compact, the cytoplasm of the

cells more abundant and intercellular fibrils more numerous. In the earlier stage the intramuscular fibroma shows a diffuse infiltration of the muscle, chiefly of inflammatory character, later the tumour is well defined but there is always a more or less definite inflammatory reaction in the surrounding zone. Regression of the tumour occurs through the combined processes of necrosis and resorption as described for the cutaneous tumour.

### *Cytoplasmic inclusions in the fibroma cells*

With special staining methods it is possible to demonstrate the presence of characteristic inclusions in the fibroma cells. Most useful in this respect is staining with anilin blue according to Mallory's method (Heidenhain's modification). With this method there appear in the fibroma cells distinct cytoplasmic inclusions in the form of blue granules. They are not visible in the earliest stages but may already be present in week-old lesions as very small granules in single fibroma cells, visible only with the oil immersion lens. The inclusions gradually increase in size and number during the development of the tumour, they appear in more and more of the cells and reach their maximal amount just before regression. At this stage the fibroma cells, as mentioned above, have a more abundant cytoplasm than before. The type of the inclusions varies: in some cells they appear as single or numerous small granules (fig 7a), in others the granules are bigger (fig 7b), while yet other cells contain still bigger homogeneous droplets, apparently originating from confluence of granules (fig 7c). Several types of inclusions may occur in one and the same cell. The inclusions do not show any typical localisation in the cytoplasm: they may appear at one or both poles of the nucleus or around the nucleus or in the peripheral cytoplasm. Nor do they show any typical arrangement apart from a certain tendency of the small granules to accumulate and collect into bigger droplets. The amount of cellular inclusion gradually increases during the development of the tumour and is therefore in some degree an indicator of the age of the fibroma. It follows from this that the inclusions are very sparse in fibroma lesions of short duration but may be seen in large amount in animals with slowly regressing tumours. In these the inclusions appear as bigger droplets, clearly visible with lower magnifications. They are consequently rather numerous in subcutaneous or intramuscular lesions, since these often persist for a longer time.

In eosin-stained sections the larger droplets are often visible through a seemingly empty halo, but they do not differ in their colour from the surrounding cytoplasm, only on rare occasions some of the droplets assume a pink colour, the smaller

INFECTIOUS FIBROMA OF RABBITS



FIG 5—Fibroma in a later stage after intracutaneous inoculation the cellular exudation is less conspicuous and the more cellular reaction is more prominent. H and E  $\times 220$

FIG 6—Enlargement of the downward projections from the epithelium overlying the fibroma several 'inclusion bodies' are visible. H and E  $\times 67$



FIG 7—Cytoplasmic inclusions in the fibroma cells (a) several small granules (b) larger granules (c) inclusions in the form of bigger droplets, filling the cytoplasm. Azocarmine Mallory  $\times 1250$



granules are not visible with this method of staining. The same applies to van Gieson stained sections, where only the larger droplets show a slight yellow-brownish colour, and even these do not stand out clearly. The inclusions are distinctly visible in sections stained with methyl violet (Weigert's fibrin stain), with which they assume a deep violet colour. Many are stained deep black with Heidenhain's iron hæmatoxylin and assume a red-violet colour with Mallory's phosphotungstic acid hæmatoxylin. With Mann's stain they are stained pale blue. Mucicarmine is negative, as are staining with Schiærlach R and impregnation with silver.

On examination of fresh preparations of fibroma tissue the cytoplasm of the fibroma cells appears filled with small granules. If the preparations are made from advanced fibroma lesions there appear amongst these granules clear, rounded, apparently homogeneous masses of a vacuole like type, which in size and distribution correspond to the inclusions observed in stained specimens. In supravital staining with neutral red they assume a brick red colour, more intense than but of the same quality as that of the smaller granules. Supravital staining with trypan blue is negative, indicating again the basophilic nature of the inclusions. Supravital staining with Janus green is negative.

Smear preparations from the tumour tissue, stained with Giemsa, do not show these cytoplasmic inclusions. The cytoplasm of the fibroma cells appears by this method to be foamy and filled with vacuoles of varying size and of a slightly paler colour than the surrounding cytoplasm. In addition there appear in some fibroma cells very small acidophilic granules and single cells may show irregular acidophilic bodies, apparently composed of the smaller granules. The acidophilic inclusions are only visible in single cells and do not show any regular occurrence. They are described by Hyde, who suggests a relation between them and the virus.

#### *IA lesions*

Andrews (1936) has given a thorough description of the lesions produced by the IA strain in the testis. After intracutaneous inoculation the IA virus produces a slight reddish papule, rather similar to the early OA lesion. It does not develop however to a circumscribed elevated nodule but remains rather flat and assumes after a few days a yellow colour, especially in its central part. With this the reaction gradually subsides, sometimes small hæmorrhages appear and after a few days the reaction is reduced to a dry, yellowish area which soon disappears. Microscopically the condition has the character of an oedematous inflammation with a gradually increasing number of leucocytes. As distinguished from the OA lesion there is scarcely any proliferation of fibroma cells and the picture is that of a purely inflammatory lesion.

*Inoculation of fibroma virus after local blockage of the  
reticulo-endothelial system*

The earlier stages of the infectious fibroma show a proliferation not only of undifferentiated connective tissue cells between the bundles of collagen but also of the histiocytes around the capillaries and to a certain extent of the endothelial cells, indicating that the action of the virus is directed against the undifferentiated mesenchyme in its widest sense. Ledingham (1927) has shown that the intracutaneous response to the vaccinia virus is modified in degree or completely abolished if the virus is inoculated in conjunction with india ink or superimposed on a site previously infiltrated with an ink suspension. In order to see if the same applied to the infectious fibroma virus, two rabbits were inoculated intracutaneously with india ink in increasing dilutions from 1/8 to 1/2048 and two rabbits with trypan blue in dilutions up to 1/256, 5 hours later the same spots were injected with fibroma virus (0.1 c.c. of a 5 per cent suspension of testis diluted 1/100). The accompanying table shows the results.

TABLE

*Delaying effect of previous injections of india ink and trypan blue on the  
development of the virus reaction*

Virus super- imposed on ink diluted	Days after inoculation						Virus superimposed on trypan blue diluted	Days after inoculation						
	5	6	7	8	9	10		6	7	8	9	10	13	
1 8	—	—	—	—	—	—	Saturated solution	—	—	—	—	+	++	
1 16	—	—	—	—	—	—		1 2	—	—	—	—	+	++
1 32	—	—	—	—	—	—		1 4	—	—	+	++	++	++
1 64	—	—	—	—	—	+		1 8	—	—	+	++	++	++
1 128	—	—	—	+	+	+		1 16	—	—	+	++	++	++
1 256	—	—	+	+	++	++		1 32	—	—	+	++	++	++
1 512	—	+	+	+	++	++	1 64	—	+	++	++	++	++	
1 1024	—	+	+	+	++	++	1 128	+	+	++	++	++	++	
1 2048	—	—	+	+	++	++	1 256	+	+	++	++	++	++	
Saline only	+	+	++	++	++	++	Saline only	+	+	++	++	++	++	
" "	+	+	++	++	++	++	" "	+	+	++	++	++	++	

A delaying effect on the virus reaction was visible in both ink and trypan-blue experiments, especially the former, where the reaction was inhibited in areas injected with the stronger ink suspensions. It seems reasonable to conclude that the functional activity of the local reticulo-endothelial system at the place of inoculation is of importance for the reaction, and in conjunction with the histological findings in the early stages of the fibroma the results may indicate a primary attack of the virus on elements belonging to this system.



cells from the surrounding medium, which would be in accordance with the origin of the cells from the undifferentiated mesenchyme with its high phagocytic power. Hyde, however, from experiments with trypan blue, denies that the fibroma cells are endowed with phagocytic properties. Finally, and most probably, the inclusions may represent degeneration products of the cell, gradually accumulating in the cytoplasm. It is possible to arrange the virus diseases in a continuous series from those in which the virus causes necrosis of the affected cells to those where the effect is cell proliferation as in the Rous sarcoma. If the infectious fibroma represents a connecting link between these two extremes the occurrence of these cytoplasmic inclusions may indicate that the effect of the virus on the cell is to cause degeneration as well as proliferation.

### Summary

The histological structure of the infectious fibroma of the rabbit shows both inflammatory and neoplastic features, the early stages are chiefly granuloma-like, whereas neoplastic features dominate the later stages. The fibroma cells originate not only from fibroblasts but also from perivascular histiocytes and endothelial cells, groups of young capillaries with hyperplastic endothelium serving as centres for the proliferation of the young tumour. The fibroma cells show characteristic basophilic cytoplasmic inclusions increasing in amount and in size with the age of the tumour. The epithelium overlying the intracutaneous fibroma shows inclusions in the form of eosinophilic granules and sometimes also hyperplasia. Regression of the fibroma occurs through a combination of necrosis and resorption, of which the former seems to be primary.

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sufficiently rare to justify the following report of a case, particularly as it presents some unusual features which may throw further light on the physiology of the pancreas

#### Case report

*Clinical history* The child, a girl of 4 years 4 months at the time of her death, had been admitted 5 months previously to an institution for mental defectives. She was an idiot, speechless, incapable of walking or even of standing by herself, helpless and without any control of her sphincters. She had been under continuous medical supervision since infancy. She had a voracious appetite with a particular liking for fruit, but in spite of this exhibited habitual constipation. Her stools had never been bulky or greasy and she had suffered no recognisable illnesses, in particular she had never been jaundiced. On admission she was found to be thin, pale and under weight, but exhibited no gross physical abnormalities or evidences of active disease.

On 27th July she had an attack of diarrhoea and vomiting which cleared up in two days. On 16th August she developed a typical attack of chicken pox. During this illness her constipation was worse and necessitated frequent enemata and, later, daily administration of mist alb. On 26th August signs of bronchopneumonia developed but soon began to clear up. Her general condition was improving steadily when on 26th September there was an abrupt rise of temperature to 102° and renewed signs of pulmonary consolidation. Two days later her bowels became relaxed and her stools are recorded as having been yellow and porridgy. On this day her bowels were opened three times and they remained rather loose for the next 5 days, after which they returned to normal and so remained until her death on 13th October. The faeces were never examined chemically, but during the period of relaxed motions the absence of specific bacterial infection was established by cultures.

*Post-mortem examination* The body was undersized and wasted, the brain small and the convolitional pattern imperfect. The pituitary gland was normal in size and appearance. The lungs contained numerous abscesses in both lower lobes, with areas of bronchopneumonic consolidation. The thymus was atrophic and the thyroid slightly reduced in size but otherwise normal. In the abdomen the most obvious feature was the extreme degree of fatty change in the liver, as can be gauged from the fact that it sank only gradually in water. No abnormalities were discovered in the alimentary canal, kidneys or adrenals.

The *pancreas* presented an unusual pale yellow, glistening, semi-translucent appearance. Its size was, if anything, larger than normal, and it possessed the usual shape and superficial lobular markings. It floated buoyantly in water and on incision it was apparent that little if any pancreatic tissue had escaped replacement by adipose tissue (fig 1). No calculi were present and Wirsung's duct was not dilated. Unfortunately the organ had been removed from the duodenal loop before the significance of the condition was appreciated and the patency of the openings of the ducts of Wirsung and Santorini into the duodenum had not been tested. A probe

ADHESIVE TISSUES OF FINE TISSUE



FIG. 1.—Section of tissue stained with hematoxylin and eosin. The connective tissue remains all the time.



FIG. 2.—Section of tissue stained with hematoxylin and eosin. The connective tissue remains all the time.



FIG. 3.—Section of tissue stained with hematoxylin and eosin. The connective tissue remains all the time.



FIG. 4.—Section of tissue stained with hematoxylin and eosin. The connective tissue remains all the time.



passed forward into the head along the duct of Wirsung from an opening made into it at the junction of the head with the body was obstructed by fibrous tissue before the plan of the common bile duct was reached. Santorini's duct similarly tested was not found to be obstructed, but as only a short length was available the condition of its duodenal termination is not known.

### *Histological examination*

The pituitary, thyroid and adrenal glands presented no abnormal features. The lungs showed typical suppurative foci in areas of acute bronchopneumonia. A typical lipoid pneumonia was found as the basis of the acute lesions. (This finding though unexpected was not surprising in view of the mental and physical state of the child, which necessitated its being fed.) The liver showed an advanced grade of fatty change (fig. 2) without any evidence of necrosis, bile stasis or infection.

**Pancreas.** A section across the head of the pancreas (fig. 3) exhibited mainly adipose tissue in which were embedded a few small collections of gland acini and minor islands of Langerhans. The largest collection of acini found in any section measured 0.7 x 0.5 mm., but most were much smaller and often consisted of two or three acini only. They were slightly more frequent around the main ducts and their branches and particularly in the upper part of the head. In the body and tail (fig. 4) gland tissue was much scarcer and often completely absent over large areas. When present the acini were nearly always attached to the islands of Langerhans. All ducts were embedded in a slightly increased amount of fibrous tissue, but nowhere else was there any evidence of fibrosis or of an excess of loose connective tissue. The vascularity of the organ was reduced. For the most part the islands of Langerhans were readily distinguishable from acinar tissue but in many places small collections of cells of indeterminate type were present. The number of islands per sq. mm. was found to be 1.37. This is somewhat higher than the figure usually found in the normal gland (0.4-1.0 per sq. mm.). A similarly increased distribution per unit area (1.76 per sq. mm.) was noted by Clarke and Halbach (1923-24) who reported the evidence of an increased total number of islands in the gland. It is probable however that their estimation was based on a few paraffin sections in which case the number may be regarded as approximate only and due to the pancreatic shrinkage which this fixation of pancreas would undergo in the course of the preparation of the sections as compared with the normal gland. It is also a little slightly more numerous in the tail than in the head but it is not clear did they appear hyperplastic as regards the total number of islands or merely condensed the islands of the organ.

most were much smaller. The average mean diameter of 50 consecutive islands was  $113 \mu$ , the range being from 42 to  $202 \mu$ . These measurements are in accord with the normal dimensions. None of the islands examined showed signs of sclerosis or hyalinisation and, though the application of Bensley's staining technique was not highly successful (autopsy not performed until 24 hours after death), the granular nature of most of the cells could be distinguished.

### *Discussion.*

From these observations there appears to be no doubt that this child had lived for some time without the aid of the external pancreatic secretion to assist in its digestive and metabolic processes. In this respect it represents the human counterpart of those animals in which the duct of Wirsung had been ligated. It is therefore of interest to see whether the case sheds any new light on the mechanism by which metabolism is effected in the absence of pancreatic ferments, and also to see to what extent, if any, the condition contributes to the early death which usually occurs in human beings so affected.

Before discussing these problems we may consider whether there is any evidence as to time at which the duct obliteration occurred and the method of its production. The fact that the fat-replaced pancreas has retained its normal configuration and size suggests that it did not occur during early foetal life. On the other hand it must be remembered that both in the experimental animal (MacCallum 1909, Boldyreff, 1935) and in some acquired cases of calculous obstruction in human adults (Dillon, quoted by Zeckwer, Zeckwer, 1934) the pancreas undergoes considerable atrophic shrinkage as well as fat replacement. Too much importance therefore should not be attached to this finding. Further conflicting evidence on this point is provided by the fact that in the case of Tiling, which appears to be an undeniably congenital example in a child of 7 months, there was pronounced atrophy and no fat replacement, whereas in Clarke and Hadfield's case, a child of 4 years in whom the condition certainly dated from early infancy, if indeed it was not of congenital origin, fat replacement was the essential and atrophy a minor feature.

In the present case therefore it is possible that the duct obstruction started soon after birth. The fact that there is no record of any illness which might be regarded as causative supports the view of a very early commencement of the duct obliteration, particularly as the continuous medical supervision necessitated by her mental state renders it unlikely that such an illness would have been missed. The absence of diffuse fibrosis of the organ such as occurs in cases of inflammatory obliteration of the duct (Valkry-Radot, Miget and Gauthier-Villars, 1933) is also in favour of an



early obliterative process, probably in the nature of a continuation of post-fatal life of a congenital stenosing or atresic process, and the youth of the child at death further strengthens this conclusion.

Our knowledge of the mechanism of metabolism in the absence of the external pancreatic ferments is based upon the experimental work of Allan, Bowie, MacLeod and Robinson (1924) and Herlevy and Soskin (1931). The former showed that depancreatized dogs on a controlled diet and in spite of controlled insulin administration died with extreme fatty degeneration of the liver in from 1 to 5 months; the latter that an adequate assimilation of proteins and fats could be maintained if large quantities of food were given and that the death of the animals could be prevented if the diet included phospholipids such as lecithin to aid the liver in its metabolism of fats. It is presumed that the proteins and fats are broken down by ferments in the succus entericus or by bacterial action within the intestine or both. To this extent therefore the present case merely confirms knowledge gained from these animal experiments. The child's voracious appetite (she had vomited what which would not be lecithin and other phospholipids in such articles as milk and eggs) and avidity for fruits must have ensured the intake and assimilation of a sufficiency of all the necessary food elements. Our case however presents one unusual feature viz. the complete absence of greasy stools which in both the other recorded congenital (or childhood) cases constituted the main clinical evidence of a disturbance of pancreatic activity. Whether this was due to an inferior superior fat splitting power of the succus entericus or to a powerfully hypolytic bacterial flora in the intestine is now unfortunately not capable of investigation.

The death of depancreatized dogs kept under controlled insulin administration (Allan *et al.*) was shown to be brought about in the same way as in the hepatectomized animals of Mann and Macleod (1923). In all the pancreaticectomized animals death occurs as hepatic failure with hypoglycaemia and at post mortem the livers are found to be extremely fatty, often containing as much as 50 to 59 per cent of their dry weight of fat. Exactly similar changes have been reported in most of the human cases of congenital or acquired pancreatic duct obstruction. Thus Lilings and Zickert's cases were hypoglycaemic before death and extreme fatty changes in the liver were outstanding features in the post-mortem findings in Clarke and Hildnehl's and Lilings' cases. In the present case the fatty change in the liver has been mentioned and the pathologic proof of hypoglycaemia before death is of more interest to the physician attending the child as reported by the ward physician. The ordered glucose administration at the age of 10 months when he felt it was "crying out for glucose." The case thus serves as a support to the views expressed by the above cited cases.

and others that hepatic failure is the potential danger in these cases. It is true that intercurrent infections have usually been present in the recorded cases and have provided convenient diagnoses for purposes of death certification, but it is highly probable that the real role of these infections is that of providing by their toxic effects the initiating stimulus to the degenerative process in the liver which, by interfering with hepatic function, leads to a progressive accumulation of fat in this organ and so to eventual repression of its power of mobilising glucose, with death in hypoglycæmia.

In conclusion it appears justifiable to suggest that while the examination of these human cases confirms the conclusion, based on animal experiments, that mammalian metabolism can be carried on effectively in the absence of the external pancreatic secretion there is now sufficient evidence to indicate that in human beings hepatic failure is an ever-present potential danger and one which is liable to be converted into a serious actuality by any lesion such as an intercurrent infection which places any further strain upon the liver. If this conclusion is accepted it follows that in all cases of this kind the effect upon the liver of any intercurrent infection should immediately be combated by the administration of glucose.

### *Summary.*

A case is described in a child of 1 years of massive fat replacement of the pancreas, with survival of the islands of Langerhans. The condition is believed to have been present from birth or early infancy. The case is discussed in the light of the results of experimental depancreatization and ligation of the main pancreatic ducts, and the conclusion is drawn that while the mechanism of maintaining metabolism is probably the same in human beings as in experimental animals, there is reason to believe that in man hepatic failure is a more serious potential complication and that the early death of most if not all of the recorded cases has been contributed to or actually caused by this complication.

I have much pleasure in acknowledging my indebtedness to the Ward and the Medical Superintendent (Dr R. Berry) of Stoke Park Colony, Bristol, for permission to make use of the records of this case, and to Dr R. Bates, F.R.C.S., for his valuable assistance in providing clinical details.

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and others that hepatic failure is the potential danger in these cases. It is true that intercurrent infections have usually been present in the recorded cases and have provided convenient diagnoses for purposes of death certification, but it is highly probable that the real role of these infections is that of providing by their toxic effects, the initiating stimulus to the degenerative process in the liver which, by interfering with hepatic function, leads to a progressive accumulation of fat in this organ and so to eventual repression of its power of mobilising glucose, with death in hypoglycæmia.

In conclusion it appears justifiable to suggest that while the examination of these human cases confirms the conclusion, based on animal experiments, that mammalian metabolism can be carried on effectively in the absence of the external pancreatic secretion, there is now sufficient evidence to indicate that in human beings hepatic failure is an ever-present potential danger and one which is liable to be converted into a serious actuality by any lesion such as an intercurrent infection which places any further strain upon the liver. If this conclusion is accepted it follows that in all cases of this kind the effect upon the liver of any intercurrent infection should immediately be combated by the administration of glucose.

### *Summary.*

A case is described in a child of 4 years of massive fat replacement of the pancreas, with survival of the islands of Langerhans. The condition is believed to have been present from birth or early infancy. The case is discussed in the light of the results of experimental depancreatisation and ligation of the main pancreatic ducts, and the conclusion is drawn that while the mechanism of maintaining metabolism is probably the same in human beings as in experimental animals, there is reason to believe that in man hepatic failure is a more serious potential complication and that the early death of most if not all of the recorded cases has been contributed to or actually caused by this complication.

I have much pleasure in acknowledging my indebtedness to the Warden and the Medical Superintendent (Dr R. Berry) of Stoke Park Colony, Bristol, for permission to make use of the records of this case, and to Dr R. Bates, F.R.C.S., for his valuable assistance in providing clinical details.

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exhaustive, were carried out with this objective. Owing to the difficulty of securing sufficient quantities of suspension from suitable cases, experiments in which a combination of suspension and streptococci or their products was used have of necessity been limited, particular attention being given to testing the possible infectivity of the suspensions.

### *Material and methods*

It was obviously of the utmost importance that the rheumatic material selected should offer the greatest chance of providing a supply of virus bodies in maximum profusion, i.e. it should be obtained as fresh as possible and from highly active cases. In rheumatic fever it had been observed that suspensions prepared from pericardial and pleural exudates contained these bodies in greatest number and in apparent purity, and for agglutination tests these were found to be the most satisfactory source of material. In a large proportion of the present experiments, therefore, these were used, but in two instances synovial membrane and joint fluid from an early acute case of rheumatoid arthritis were employed.

The technique used in the preparation of suspensions has been described elsewhere (Eagles *et al.*) and with slight modification was used in infection experiments. The whole deposit obtained by high-speed centrifugation of exudates, from which cells and debris had previously been removed, was incorporated in either normal saline or the supernatant fluid of the specimen from which it had been obtained. Washing the deposit and differential centrifugation were not carried out. In some instances exudates were injected without previous manipulation. Nodules were macerated in saline and the supernatant fluid used after removal of cellular debris by slow centrifugation (3000-4000 *r.p.m.*). Fluid for injection was obtained from synovial membrane from rheumatoid arthritis and from fibrin either by maceration and grinding or with the aid of a tissue press. Cerebrospinal fluid from fatal cases of rheumatic fever was also utilised.

As far as possible material for injection was obtained from active cases of undoubted rheumatic disease and when these were fatal the diagnosis was confirmed *post mortem*. As short a time as possible elapsed between procuring the specimens and animal inoculation but this was of necessity variable. Suspensions were tested for bacterial growth but the inoculations were carried out before the results were available. They were also examined by dark ground illumination and in stained film preparations for the presence of virus-like bodies. In a number of instances agglutination tests were made with a portion of the suspension after suitable manipulation to secure even dispersion of the particles.

### *Animal experiments*

The choice of a suitable experimental animal which might prove susceptible was difficult, particularly in view of the fact that most laboratory animals have at one time or other been unsuccessfully tested with rheumatic materials. Some indication of susceptibility to a naturally occurring type of articular rheumatism has been described in domestic animals by Hutyna and Marek (1917) but it is not certain that the infection is strictly comparable to the human disease. Cattle are most frequently affected but it occurs also in

horses, swine, goats and sheep and very rarely in dogs. In cattle the conditions under which they are kept seems to exert a predisposing influence, the disease appearing only in animals confined indoors over long periods. Grazing animals are very infrequently affected. In addition to joint inflammation, verrucose and ulcerative endocarditis, sero-fibrinous pericarditis and peritonitis are described. The disease is said by these authors to be favourably influenced by salicylates. It would appear, therefore, that these animals should be suitable for the experimental production of rheumatic disease and have been so used by some investigators. Their unsuitability in our case lay in the difficulty of maintenance under laboratory conditions and in the fact that sufficiently large quantities of inoculum were not available. We have used instead young *Macacus rhesus* monkeys in their first dentition and in one instance a young *Macacus cynomolgus*, because of their generic relationship to the human species, the ease with which they may be procured and maintained and their proved susceptibility to experimental infection with a number of viruses affecting man.

Inoculations were given intrapericardially, intraperitoneally, intratracheally, intramuscularly, into the joint cavities, into the nasal mucosa and, on one occasion, intravenously. Intrapericardial injections were given under full anaesthesia and with thoracotomy. By this procedure it was possible to secure the parietal pericardium with forceps and ensure that the inoculum was given into the pericardial space. The operation was tolerated surprisingly well, the animals being perfectly well and active in a few hours. The wound healed by first intention and, *post mortem*, little trace of the operative procedure was evident.

We have attempted to produce infections with rheumatic suspensions and materials alone and also in conjunction with streptococci and streptococcal products, mainly toxin. The toxin used was of high toxic value and was prepared by the Burroughs Wellcome Laboratories by the method of O'Meara (1934), using the strain Dochez, N Y 5. It was, therefore, predominantly exotoxin.

Animals were observed over a period of several months for clinical signs of infection. Frequent electrocardiographic examinations were made and in recording these the same abbreviations have been used as for the normal human curve. Temperature observations were not recorded throughout because these were found to be extremely variable even in normal control monkeys, which made their interpretation difficult and of doubtful value. Erythrocyte sedimentation rates (B S R) were observed in most instances, the method of Payne (1932) being employed, except that in our experiments venous blood was used. At varying periods following inoculation animals were killed, *post mortem* examinations made and sections prepared.

## RESULTS

**Monkey no. 1.** *M. rhesus* 22 11 35; deposit from 20 c.c. pericardial exudate from fatal rheumatic fever injected intrapericardially in 1 c.c. saline. Suspension contained numberless bodies of uniform size and density which agglutinated specifically with rheumatic sera. Under observation for several months and remained perfectly well.

**Monkey no. 2** *M. rhesus*. 22 11 35, deposit from 15 c.c. pericardial exudate from same case suspended in 1 c.c. saline. 0.5 c.c. injected submucously in right and left nostril. Deposit from 15 c.c. of same fluid suspended in 1 c.c. saline injected into trachea. Remained perfectly well until 13 12 35, when cough and diarrhoea developed. This condition continued with increasing weakness until 23 12 35, when animal was killed *in extremis*. *Post mortem*, the heart appeared perfectly normal. The lungs showed evidence of widespread acute bronchopneumonia.

**Monkey no. 3** *M. cynomolgus* 10 12 35, deposit from 7 c.c. arthritic fluid from rheumatic fever injected intrapericardially in 1 c.c. saline. Suspension showed numberless bodies of uniform size and density. Remained well until 3 or 4 days before death on 7 1 36 from bronchopneumonia.

*Post mortem*, the heart showed no abnormality macroscopically or microscopically.

**Monkey no. 4** *M. rhesus* 18 12 35, deposit from 20 c.c. pericardial fluid from fatal case of acute rheumatic fever injected intrapericardially in 1 c.c. saline. Suspension showed numberless bodies of uniform size and density which were specifically agglutinated by rheumatic fever serum. Observed for several weeks and remained perfectly well.

**Monkey no. 5** *M. rhesus* 20 12 36, deposit from fibrin obtained from pericardium of case used for monkey no. 4 injected intrapericardially in 1 c.c. saline. Numberless bodies similar to those from the fluid, they were specifically agglutinated by rheumatic fever serum. Observed for several weeks, remained perfectly well.

**Monkey no. 6** *M. rhesus* 7 5 36, deposit from 10 c.c. joint fluid from subacute rheumatoid arthritis injected into left knee in saline. 11.10.36, deposit from 20 c.c. pericardial fluid from fatal rheumatic pericarditis injected intrapericardially in 1 c.c. saline. 19 10.36, 0.5 c.c. scarlet fever toxin injected subcutaneously. 28 10 36, 5 c.c. scarlet fever toxin injected subcutaneously. 3 12 36, 24-hour culture in rabbit's blood broth of streptococci from pharyngitis in a rheumatic child sprayed into throat. (The pharyngitis was not followed by a recurrence.) Observed until July 1937, remained well except for the development of rickets. Nine estimations of B.S.R. between February and July 1937, all normal. Four electrocardiograms (E.C.G.)\* showed no significant changes. 20 8 37, still well, killed.

*Post mortem*, no abnormality seen. Sections stained with H and E. In heart one small aggregation of lymphocytes in connective tissue in ventricular muscle. Valves, aorta and other organs normal.

**Monkey no. 7** *M. rhesus* 19 10 36, 0.5 c.c. scarlet fever toxin injected subcutaneously. 28 10 36 and 28 11 36, 5 c.c. scarlet fever toxin injected subcutaneously. 19 12 36, E.C.G. P-R = 0.9; Q-R-S = 0.1, rate 240 per min. 20 1 37, suspension from cerebrospinal fluid removed.

\* The abbreviations used are those of the normal human curve, namely —

P deflection = auricular systole

Q-R-S deflection = spread of impulse through the Purkinje system

T = end of ventricular systole and coincides with the second sound

Time factors — P-R interval = beginning of P to beginning of R



*post mortem* from case of rheumatic corditis with nodules injected intrapericardially in 1 c c saline. Observed until July 1937, 8 B S R estimations were normal and the animal appeared to be well. During this period three additional electrocardiograms were recorded 3 4 37; P R = 00 Q R S = 04, rate 240 per min 8 5 37, P R = 00, Q R S = 01, rate 200 per min 17 7 37, P R = 00 Q R S = 04, rate 200 per min 26 8 37, still well, killed.

*Post mortem*, no macroscopic abnormality. Sections from heart, lung, liver, spleen and kidney stained with H and E. In heart traces of degenerate muscle near cusps of left ventricle and sparse infiltration with mononuclear cells. One perivascular lymphatic full of lymphocytes seen in ventricular muscle. No other abnormalities. Other organs normal.

Monkey no 8 *M. rhesus* 14 5 36 supernatant fluid from rheumatic nodule centrifuged at 3000 1000 r p m after maceration and the addition of saline injected intrapericardially 11 10 36, deposit from 20 c c pericardial fluid from fatal rheumatic pericarditis injected intrapericardially in 1 c c saline. A Gram negative bacillus (probably *B. proteus*) was found on culture of suspension 10 12 36 noticed to be unwilling to extend legs, looked ill no clinical abnormality of heart or lungs. 18 12 36, serum agglutinated suspension from rheumatic pericarditic fluid in titre of 1 8. Weakness, dyspnoea with grunting respirations pallor and slight cyanosis became gradually more severe 23 1 37, ECG recorded but syncopal death occurred while further records were being taken. Three records were obtained. The first showed well marked sinus arrhythmia, Q R S small and slurred, T<sub>1</sub> and T<sub>2</sub> inverted T<sub>3</sub> flat, P R = 00 rate 200 per min. In the second there was some closing of the R T interval in lead 1 and depression of the R T interval in lead 2 such as is seen in coronary disease and also sometimes during acute rheumatic carditis. In the third, taken just before death, the rate had fallen to 60. No P waves were evident in lead 2, possibly some auricular fibrillation with a slow ventricular rate. The tracing in lead 3 was too much obscured by somatic muscle movements to permit of interpretation.

*Post mortem*, no abnormality in any organ other than fine adhesions between left pleura and the chest wall. No pericardial adhesions and no excess of pericardial fluid. Blood and pericardial fluid sterile. Sections from heart, lungs, kidney, spleen and adrenal gland fixed in Zenker's osmium and stained with H and E. Heart showed a mild myocarditis, particularly of the auricle, with some loss of muscle striation, granularity of muscle fibres, small areas of fibroblastic proliferation with a few lymphocytes, some small vessels filled with polymorphs and some areas of rather sparse leucocytic infiltration. Between the endothelial and fibrous layers of the auricular endocardium there were some fibrin and leucocytes. In sections stained with Mallory's phosphotungstic acid haematoxylin and with H and E some oedema appeared in the auriculo ventricular adipose ring, in the auricular pericardium and in the auricular myocardium near the adipose tissue and throughout the ventricular myocardium. The periphery of the oedematous area in the adipose tissue showed slight infiltration with lymphocytes, plasma cells, epithelioid histiocytes and monocytes and a slight increase of fibrocytes. In the pericardium the oedematous area also showed slight infiltration with lymphocytes, granular leucocytes and plasma cells. Throughout the myocardium there was increase of fibrocytes, epithelioid histiocytes and lymphocytes. Sections stained with van Gieson showed widespread but patchy increase of collagenous fibrils in the myocardium. In the myocardium "banded" nuclei were very numerous in the endothelial cells lining capillaries and in the fibrocytes of the interstitial tissue.

**Monkey no 9** *M. rhesus*. 15.10.36, 1 c.c. of a light suspension of the broth culture of the proteus-like organism grown from the suspension injected into monkey no 8 injected intrapericardially. Inactive and looked ill persistently after the operation 19.12.36; killed

*Post mortem*, a few soft adhesions between thoracic sac and left lung; parietal pericardium thick and opaque, fairly firmly adherent to visceral layer over whole surface. The proteus-like organism was recovered from the pericardial fluid

**Monkey no 10** *M. rhesus* 10.1.36, deposit from 18 c.c. pleuritic fluid from acute rheumatic fever injected intrapericardially in 0.5 c.c. saline. Remained well. On 19.10.36 1 c.c. and on 28.10.36 3 c.c. scarlet fever toxin injected subcutaneously. 10.11.36, left wrist slightly swollen and held stiffly. 19.11.36, slight dyspnoea. 23.11.36, more dyspnoea, left wrist still appears painful. 28.11.36, symptoms more severe, appeared to have pain and stiffness of other limb; gallop rhythm on cardiac auscultation. 1.12.36, skingram of chest shows no definite enlargement of heart, mottling in lung fields suggestive of congestion or bronchopneumonia. 8.12.36 E.C.G. rate 260 per min., QRS rather wide; P-R = .09; T<sub>1</sub> inverted, T<sub>2</sub> flat, one ventricular extrasystole. 18.12.36, serum did not agglutinate suspension from rheumatic pericarditis. 19.12.36, E.C.G. rate 240 per min., P-R interval .09, QRS more slurred and slightly wide (.06), T wave in lead I inverted. 16.1.37; E.C.G. rate 220 per min., P-R interval .09, T wave in lead I inverted, QRS = .04, slight improvement in general form of curve. 21.1.37, serum did not agglutinate suspension from rheumatic pericarditis. 23.1.37, E.C.G. rate 240 per min., P-R interval .09, ventricular complexes wide, T wave in lead I inverted, QRS = .06. Clinical condition unchanged. 1.2.37, clinical condition considerably worse, killed

*Post mortem*, no macroscopic abnormality seen in any organ. The heart showed no obvious dilatation, no increase in pericardial fluid, no fibrin and no adhesions. Endocardium and valves appeared normal. Sections fixed in Zenker's acetic and stained with Mallory's phosphotungstic acid hematoxylin and eosin and by van Gieson's method. Aorta showed no abnormality except two small hemorrhages in adventitia. Aortic valve appeared normal. Auricular myocardium showed fragmentation of muscle fibres and granular degeneration which was widely spread and severe in places. This was much more pronounced than in monkey no 8. In the myocardium "banded" nuclei were present in the endothelium of the capillaries, in fibrocytes and free epithelioid cells in the interstitial tissue and in a myocardial muscle fibre. The adipose tissue of the auricular ventricular ring consisted almost entirely of cells with a homogeneous or foamy cytoplasm. The ventricular muscle fibres showed much less degeneration than the auricular, although an undoubted infiltration with epithelioid cells, monocytes, lymphocytes and an occasional plasma cell was present. In the pericardium there was an increase of fibrocytes, and a group of large lymphocytes was seen. The endothelial cells lining the capillaries appeared larger and more numerous than in sections from control monkeys. Sections stained with van Gieson showed some increased density of collagen fibres in both auricular and ventricular myocardium and in interauricular ventricular septa.

**Monkey no 11** *M. rhesus* 19.10.36, 28.10.36 and 28.11.36, 5 c.c. scarlet fever toxin subcutaneously. 21.1.37, serum did not agglutinate suspension obtained from rheumatic pericarditis fluid. Monkey remained well. Six injections of B.S.R. normal. 1.5.37; synovial membrane in a normal condition, synovial membrane, ground with sand, mixed with saline and

centrifuged at 3000 4000 rpm, 2 cc of supernatant fluid injected intratracheally and 5 cc intramuscularly. Observed for 2 months, remained well. Thrombocyte estimations normal. Three electrocardiograms showed no abnormality. 31 8 37, rachitic but no other sign of disease killed.

*Post mortem*, lungs showed dark grey areas of collapse and fibrosis in each middle lobe, left lower and right upper lobe. Sections from other organs (H and I) appeared normal.

Monkey no 12 *M. rhesus* 28 10 36, 0.25 cc of 24 hour culture of *Dochter streptococci* mixed with 5 cc plain agar and injected subcutaneously into left thigh. Leg became swollen but subsided after a few days. 13 1 37 4 cc pericardial fluid from rheumatic pericarditis injected intrapericardially. 14 1 37 found dead in morning.

*Post mortem*, collapse of both lungs, slight pericardial distension by injected fluid, spleen enlarged. The pericardial fluid used for injection showed on culture a profuse growth of haemolytic streptococci.

Monkey no 13 *M. rhesus* 28 10 36 5 cc scarlet fever toxin injected subcutaneously. 28 11 36 10 cc scarlet fever toxin injected intramuscularly. 17 12 36 serum did not agglutinate suspension from rheumatic pericardial fluid. 16 2 37 BSR normal. 20 2 37 ECG QRS well formed and fairly high. PR = 0.8, T flat in all leads. QRS = 0.4, rate 200 per min. 2 3 37 nasal discharge, cough, dyspnoea and poller. 5 3 37, found dead.

*Post mortem*, congestion and consolidation of lungs, spleen enlarged, possibly slight excess of pericardial fluid.

On section lungs showed some consolidation, with polymorphic infiltration especially dense around vessels. Liver showed some leucocytic infiltration in a few portal tracts. Supravivals also infiltrated. Heart sections stained with Mallory's phosphotungstic acid haematoxylin and eosin showed great congestion of venules and a few haemorrhages into intermuscular septa, endocardium and pericardium. Muscle fibres showed some evidence of degeneration but less than in monkeys 8 and 10. There was undoubtedly a slight infiltration of the myocardium with epithelioid cells, monocytes, lymphocytes and on occasional plasma cell. Some increase in fibrocytes. Endothelial cells lining capillaries were larger and more numerous than in control monkeys but less striking than in monkey no 8 (rheumatic material only). With von Gieson there was a slightly increased density of collagen fibrils in the intermuscular septa but for less definite than in monkey no 8. "Bonded" nuclei were present in endothelial cells lining capillaries, in fibrocytes of the septa and in a septal nerve and a myocardial muscle fibre.

Monkey no 14 *M. rhesus* 21 11 36 2.5 cc joint fluid from patient with subacute rheumatism injected into the right knee. 28 11 36 5 cc scarlet fever toxin intramuscularly. 30 11 36, deposit from acute rheumatic joint fluid injected intratracheally. 20 1 37 deposit from cerebro-spinal fluid from fatal rheumatic carditis with nodules injected intraperitoneally in 1 cc of same cerebrospinal fluid. Subsequently observed for 6 months, remained well. Nine BSR estimations were normal as were three electrocardiograms. 26 8 37, killed.

*Post mortem*, no macroscopic abnormality.

Sections (H and I) showed slight myocardial degeneration and fragmentation of muscle fibres. Liver, lung, spleen and kidney showed no abnormality.

Monkey no 15 *M. rhesus* 21 11 36, 2.5 cc joint fluid from same case as monkey no 14 (21 11 36) injected into left knee. Subsequently

observed for 8 months, remained well. Seven BSR estimations were normal 20 3 37; ECG good  $T_2$  and  $T_3$ , P-R = 0.8, rate 240 per min, QRS = 0.4 24 1 37, ECG no change except smaller deflections in lead 2, P-R = 0.9, QRS = 0.4

Monkey no 16 *M. rhesus*. 26 11 36, deposit from 20 c.c. fatal rheumatic pericarditic fluid injected intrapericardially in 1 c.c. normal saline. 28 11 36 5 c.c. scarlet fever toxin intramuscularly 3 12 36, streptococcal culture (same as that used for monkey no. 6, 3 12 36) sprayed into throat. Observed until May 1937, remained well. Six BSR investigations normal 8 5 37; 15 c.c. rheumatic pericardium fluid intravenously. Observed for 1 month remained well. Two BSR estimations normal. Three electrocardiograms showed very flat T waves in all leads.

Monkey no 17 *M. rhesus* 26 11 36, deposit from 10 c.c. of same fluid as used for monkey no. 16 (26 11 36) and from 10 c.c. cerebrospinal fluid from same case injected intrapericardially in 1 c.c. pericardial fluid from same case 18 12 36 and 21 1 37, serum did not agglutinate suspension from rheumatic pericarditic exudate. Three electrocardiograms showed very flat T waves in all leads 31 8 37; well; killed.

*Post mortem*, no macroscopic abnormality seen. Sections stained H and E. In heart two small patches of aggregated cells (lymphocytes) and one area of perivascular lymphocytic infiltration in ventricular muscle. Liver, lung, kidney, spleen and suprarenal apparently normal.

Monkey no. 18 *M. rhesus* 3 12 36, streptococcal culture (as for monkeys nos. 6 and 16 on 3 12 36) sprayed into throat. No obvious effect. 26 1 37, 3 c.c. supernatant fluid from maceration of synovial membrane, early acute rheumatoid arthritis, after centrifugation at 3000-4000 for 15 mins, injected intratracheally and 2 c.c. into left knee joint. Observed for 6 months, remained well. BSR 1 100 on 16.2 37, on 2 3 37 rose to 5 100, fell to 1 100 on 16 3 37 and 30 3 37, rose to 9 100 on 15 1 37, fell to 2 100 on 4 5 37 and 18 5 37 and 1 100 on 3 6 37 (BSR in normal monkey 3 min in first hour). Three electrocardiograms showed no significant changes 9 9 37; slightly rachitic but otherwise well, killed.

*Post mortem*, no macroscopic abnormality. Sections from heart, lung, liver, spleen, kidney and both knees (H and E) showed no abnormality other than slight rachitic changes.

Monkey no 19 *M. rhesus*. 19 5 36, deposit obtained after maceration and light centrifugation of rheumatic subcutaneous nodule injected intratracheally, remained well 4 2 37, deposit from 25 c.c. rheumatic pericarditic fluid injected intrapericardially in 1 c.c. of the fluid. 11.2 37; BSR 8 100 2 3 37, BSR 5 100 16 3 37, 30 3 37, BSR 1 100. 15 1 37, BSR 7 100 30 4 37 BSR 1 100. Early May 1937, looked ill, pallid hobbling as though hind legs were painful, but BSR 2 100 and recovery soon occurred. BSR estimations and clinical observations during the next 2 months revealed no abnormality. Five electrocardiograms showed no significant changes. 9 9 37; rachitic but otherwise well; killed.

*Post mortem*, no obvious abnormality. Sections stained H and E. Heart showed some increased cellularity, mainly of lymphocytes, with an occasional patch of cells consisting of lymphocytes, epithelioid cells and an occasional plasma cell. Increased cellularity around capillaries in pericardium and endocardium occurred in scattered areas. Lung, liver, spleen and kidney normal. Left knee—slight rachitic changes.

Monkey no 20 *M. rhesus* 1 2 37, deposit from 80 c.c. fluid from case used for monkey no. 19 (4 2 37) injected intrapericardially. Observed for 2 1/2 months; 4 BSR estimations normal. Three electrocardiograms showed no significant changes.

Monkey no 21 *M. rhesus* 30137, deposit from rheumatic pericardial fluid injected intrapericardially in 1 c.c. of supernatant. A further 11 c.c. of supernatant fluid inoculated intramuscularly in legs. Observed for 6 months, remained well. Seven BSR estimations normal. Four electrocardiograms showed no significant changes.

Monkey no 22 *M. rhesus* 4337, 0 c.c. joint fluid from rheumatoid arthritis injected intrapericardially, remained well. BSR normal until 18.5.37, when it rose to 18.100. Three electrocardiograms showed no significant changes. This monkey has remained well up to the present time (Dec. 1937).

### DISCUSSION

In spite of numerous attempts with bacteria thought to have aetiological significance in rheumatism and with oxalates and other materials from selected cases convincing evidence of experimental rheumatic infection in animals has not been produced and accordingly the causative agent or agents remain unidentified. This may perhaps be explained on the grounds that the clinical disease occurs only in man and that laboratory animals are insusceptible, that the infecting agent is supplemented by complex accessory factors such as metabolic imbalance or hereditary predisposition which cannot be reproduced at will or that the true infective agent or combination of agents has not yet been found. Reproduction of the disease is further complicated by the diverse manifestations of the rheumatic state so that it becomes necessary to have some clear idea of the essential criteria for experimental purposes. The most important and convincing evidence of acute rheumatic fever lies in the demonstration of Aschoff nodes in the affected tissues, since these occur in no other disease. Their presence along with non-bacterial pericarditis and non-bacterial verrucous endocarditis is generally regarded as signifying undoubted acute rheumatic infection. Certain clinical features are also significant, such as the signs of carditis and pericarditis with certain abnormalities in electrocardiographic tracings, subcutaneous nodules and increased erythrocyte sedimentation rates. In no instance have these criteria been satisfied experimentally.

Gross, Loewe and Eliasoph (1929), as judged on pathological evidence, failed to reproduce the disease in a variety of animals, including rabbits, guinea pigs, dogs, swine, sheep and calves, injected with a variety of pathological materials and in various ways. Most of the rabbits and all the dogs, cats and guinea pigs were full grown. Sheep were approximately eight months old and swine and calves had been recently weaned. Calves, sheep and swine were selected largely because it was thought that the vascularity of the valves in these animals might predispose to localisation of the infective agent in these areas. Amongst other materials, streptococci isolated from the blood of unquestioned rheumatic fever cases, as well as whole blood, plasma, serum, pericardial, pleural and hydrocele fluid, filtrates from tonsils, subcutaneous nodes and lymph nodes and nasopharyngeal washings were used. They realised that diversity of interpretation may exist in the

recognition of Aschoff nodes and insisted upon the presence of non-bacterial verrucose endocarditis and non-bacterial pericarditis as additional criteria of rheumatic infection. In a considerable number of animals focal accumulations of inflammatory cells, at times closely resembling Aschoff bodies, were found, but these were sufficiently distinct for the authors to have no hesitation in saying that they had failed to produce the typical Aschoff body. In none of their experimental animals were non-bacterial verrucose endocarditis or non-bacterial pericarditis encountered.

Miller (1924 *a* and *b*) observed focal perivascular collections of cells with single and multiple nuclei in guinea-pigs and rabbits in the course of an attempt to produce rheumatic manifestations with blood, serum, joint fluid, pleural exudate, filtered throat washings and tonsillar material from cases of acute rheumatism. Similar lesions were found in control animals. Sixty per cent of a group of 34 apparently healthy adult rabbits showed in sections of heart muscle lesions consisting of lymphocytes, endothelial leucocytes, occasional polymorphonuclear eosinophils, plasma cells and fibroblasts. In their test animals only two instances of an acute non-bacterial arthritis were encountered.

Swift (1935-36), using a variety of materials from undoubted rheumatic sources, also failed to find evidence of rheumatic infection in laboratory animals, including monkeys, despite previous conditioning of the animals by various methods planned to predispose them to subsequent virus infection.

In our own experiments nothing more than focal collections of inflammatory cells, myocarditis, degeneration of myocardial fibres and in two instances some increase in collagen, could be found. In no instance were multinucleated cells encountered and although the collections of cells contained elements also found commonly in Aschoff's nodules, these bore little resemblance to the typical picture seen in rheumatic heart disease. The myocarditis is of some interest since, with an accompanying proliferation of inflammatory cells, it was present in a number of the monkeys in this series. In no 8 (suspension alone) and no 10 (suspension *plus* streptococcal toxin) it was moderately severe. A similar myocarditis occurred in monkey no 13 (toxin alone), which prevents definite conclusions being drawn, the more so in that in a series of 9 control monkeys some degree of myocarditis with increased cellularity was found in three instances. It is possible that myocarditis of a chronic type is due to some common infection of laboratory monkeys, or that monkeys are liable to a myocarditis in various infections which in man would be complicated by pure myocardial degeneration. Spontaneous myocarditis has been demonstrated by Fox (1923) in twelve primates out of 498 examined *post mortem*. In our animals the valves, endocardium and pericardium remained normal, even in nos 8 and 13 where the clinical picture was suggestive of grave cardiac damage, moreover in these two animals the degree of myocarditis present in sections was insufficient to account for the clinical state. This grave condition has not hitherto been noted with any frequency in laboratory monkeys under observation in

other studies in this Institute and its nature is obscure. The pathological changes suggested the possibility of a mild degree of scurvy and bore some resemblance, though the lesions were much less severe, to those described in scurvy and carditis in guinea-pigs by Taylor (1937). In our animals, however, apart from slight oedema in one or more joints, with some tenderness, no clinical signs of vitamin C deficiency could be detected. It is surprising also that on a diet common to all the monkeys under observation over many weeks or months only isolated examples should occur. In man, apart from the typical lesions of rheumatism, pyæmia and syphilis, myocarditis is very rare, although a slight infiltration with lymphocytes, plasma cells, mononuclears and occasional neutrophils and eosinophil leucocytes, accompanied by a proliferation of fibrocytes, is relatively common in diphtheria. In the monkeys in our series where streptococcal toxin was injected the cellular infiltration observed may have resulted from mild damage by the toxin.

It has been pointed out earlier that in clinical rheumatic disease considerable importance is attached to the electrocardiographic tracings in assessing the degree of carditis present. Prolongation of the P-R interval beyond normal limits co-existing with auscultatory signs such as softening of the first sound is considered of great diagnostic value. In monkeys the normal heart rate appears to be about 210 per min under ordinary conditions of examination, which makes auscultation of little value. Electrocardiographic examination of monkeys either in health or in disease does not appear to have been made hitherto, or at least not to any extent. It was felt that tracings of inoculated animals might yield evidence of carditis should it be present. No difficulty was experienced in obtaining excellent records. An average normal was obtained by taking tracings of normal monkeys before inoculation. These were found, apart from the increased rate, to bear a very close approximation to the normal human tracing. In addition each monkey's tracing before inoculation was regarded as normal for that animal and subsequent deviations following inoculation as representing departures therefrom. The average normal P-R interval for the *rhesus* monkey appears to be about 0.8 sec when the heart rate is approximately 240. A P-R interval of more than 0.8 sec was observed only in monkeys following injection with toxin alone (M 7), and subsequently with rheumatic material or with toxin plus rheumatic material (M 8, M 10, M 15). In these instances the interval was prolonged to 0.9 sec. It is probable that this apparent prolongation is without real significance and may be within normal limits for the monkey. It does not appear to be associated with streptococcal toxin only since it occurred also when rheumatic material alone was injected. Nor is it a concomitant of the operative interference. The significance of the inversion of the T waves in leads 1 and 2 in monkey





present in any of the animals examined *post mortem*. Subcutaneous nodules were at no time present.

Admittedly the series is not large and the experiments were confined practically to one kind of monkey, but within these limits no evidence has been obtained that the suspensions of these bodies possess infectivity. Similar conclusions have been reached by other investigators, although in their experiments no attempt was made to concentrate the virus like bodies presumably present in the materials used in order to produce infection by massive doses. Further work may give some indication of the conditions governing liability to rheumatic infection or explain the nature of the agglutination of these particles by rheumatic sera, but at present infection experiments have not strengthened the opinion that the agglutination is unquestionably the result of union of virus and antibody from rheumatic sources. The position, however, is not dissimilar to that arrived at in connection with herpes zoster and chicken pox and the elementary bodies associated therewith (Ames, 1933, 1934).

Our thanks are again due to the medical staffs of many hospitals for placing their cases and clinical material at our disposal. The electrocardiograms have been recorded throughout by Miss L. Josephine Hunt, cardiographer at King's College Hospital. To her and to the department of cardiology of that hospital we are greatly indebted. Our thanks are also due to Sir Maurice Cassidy and Sir Thomas Lewis for advice in the interpretation of a number of the electrocardiographic tracings, and to Professor Turnbull and Dr E. H. Creed for opinions on pathological sections. The research has been in part carried out with the aid of a grant to one of us (G. H. L.) from the Medical Research Council, to whom our thanks are due.

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counterstain. In some cases other stains were also used, more particularly for the connective tissues, but without yielding additional information.

In the earlier experiments the hydrocarbons were dissolved in benzene, later it was found that acetone does not give rise to the changes which benzene alone produces in mouse skin, and a series of observations was made using acetone as the solvent. In general, 0.3 per cent solutions were used, but in the case of 3:1-benzpyrene observations were also made with a 1 per cent solution in benzene, and certain compounds which are not soluble to the extent of 0.3 per cent in acetone were used as saturated solutions in this solvent (1:2:5:6-dibenzanthracene, 1:2:5:6-dibenzacridine, chrysene).

The hydrocarbons used and their sources were as follows —

From Rocho Products, Ltd (with a small sample also from Professor Cook)—methylcholanthrene

From L. Light & Co and Professor Cook—3:4-benzpyrene.

From the Organic Chemistry Department, the University of Leeds—1:2:5:6-dibenzanthracene.

From Professor J. W. Cook—cholanthrene, 1:2:5:6-dibenzacridine, 3:1-benzphenanthrene, 1:2-benzanthracene, chrysene, pyrene, phenanthrene, 3-methyl-1:2-benzanthracene, 7-methyl-1:2-benzanthracene, 1:9-dimethylphenanthrene, 1:2:3:4-dibenzanthracene, fluorene, 1:2-benzfluorene.

It early became apparent that one of the most interesting differences between the more potent carcinogens and the other compounds was the greater depilatory activity of the former, so a special experiment was undertaken to investigate this phenomenon.

## EXPERIMENTAL OBSERVATIONS

### *Macroscopical changes.*

**Epilation.** A week after the first application of a potent carcinogen, a high proportion of the mice are completely epilated over the treated area of skin. The skin is smooth and only rarely shows any trace of ulceration or excoiation, and when these occur they may be attributed to some coincident agent, e.g. infection or parasites. The surface of the bald skin is matt, not shiny, it sometimes gives the impression on pinching it that it is slightly thickened as if by oedema; in colour it is perhaps slightly ruddier than normal, though a comparison as to colour between the normal and the epilated skin is not altogether satisfactory.

The rates of epilation were in the same order as those of tumour induction for the four carcinogens methylcholanthrene, cholanthrene, 3:1-benzpyrene and 1:2:5:6-dibenzanthracene. Thus with the first two, all the mice showed an area of complete alopecia within two weeks, with 1:2:5:6-dibenzanthracene three weeks on an average was required to reach this stage, while 3:1-benzpyrene was intermediate in effect in 0.3 per cent. solution, and about equal to methylcholanthrene (0.3 per cent.) when used in 1 per cent solution in benzene. In all cases epilation was more rapid

with benzene than with acetone solutions. The weak carcinogens 3-methyl-1-benzophenanthrene and 1,2,5,6-dibenzanthracene showed a depilatory rate no greater than that of some of the non-carcinogens and substantially less than that of two of them (3- and 7-methyl-1,2-benzanthracene)\*.

The state of complete alopecia does not last long and the regrowth of hair takes place in a time roughly proportionate for each substance to the time taken to depilate. With methyl-1-benzanthrene, for example, some of the mice show regenerated hair at the end of two weeks at a time when other similarly treated animals are still bald. The new hair can usually be easily distinguished from the old as it has a scrubby appearance and is lacking in the normal curves which give the characteristic look appearance to mouse fur as a whole. With 7-methyl-1,2-benzanthracene (the most active depilator of the non-carcinogens) hair loss is considerably less pronounced and at 16 days it is yet unaccompanied by any regeneration.

None of the non-carcinogenic hydrocarbons produced complete alopecia in the early weeks and only rarely did so at any stage of the experiments which were continued for periods of at least 21 weeks. There was also a lower activity in hair regeneration so that the condition of the fur of individual animals did not show the striking alteration from one week to the next which was so characteristic of animals treated with the potent carcinogens. This was an important difference between the action of say, methylcholanthrene and 7-methyl-1,2-benzanthracene, and is emphasised because this aspect of the phenomenon is marked in figs 1 and 2, in which an attempt has been made to give a graphic indication of the quantitative depilatory relationship of the various substances used. It will be obvious that if a group contained two mice, one completely epilated and one fully coated which displayed the reverse conditions a week later, the graph would show a resultant value of 50 per cent epilation for both weeks and would be the same had each mouse been half epilated at each observation.

Nevertheless in spite of concealing this element of difference between the potent carcinogens and the non-carcinogenic analogues, figs 1 and 2 serve to show that the depilatory activities of methylcholanthrene, chrysanthrene, 1-benzopyrene and 1,2,5,6-dibenzanthracene are significantly superior to those of the other hydrocarbons tested. This is especially true of fig 2 where the results with a strong sedative are recorded. The

\* Professor C. W. C. has pointed out that the depilatory activity of the carcinogens probably can be estimated more precisely by calculating the percentage of 3-methyl-1,2-benzanthracene. This is of interest as H. H. A. and H. H. A. (1957) had found that 3-methyl-1,2-benzanthracene had a greater depilatory activity than that of the non-carcinogenic hydrocarbons.

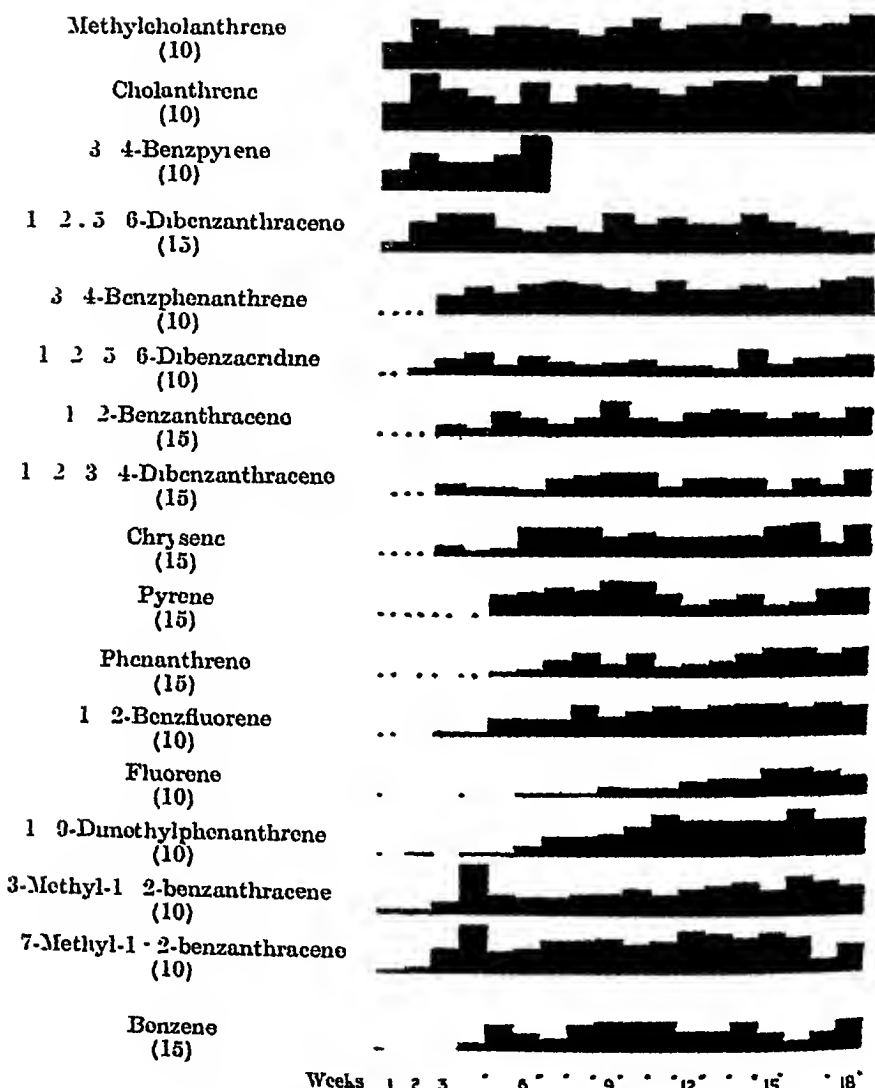


FIG 1 —Depilatory effect of various carcinogenic and non carcinogenic hydrocarbons (in benzene).

The degree of alopecia was estimated in each mouse each week as very slight, slight, moderate, considerable, marked or complete. To these were given the values 1 to 6 respectively, and for each week the numbers were added up and expressed as a percentage of the maximum possible value for the group. *E.g.*, if in a group of 10 mice, 3 were normal, 4 slightly epilated, and 3 considerably epilated, the result would be  $(3 \times 0) + (4 \times 2) + (3 \times 4) = 24$ , which expressed as a percentage of  $(10 \times 6)$  is 40. The values so obtained are those plotted, and while it is realised that such a representation of the data smoothes out each group and obscures the rapid and striking changes in the hair of single mice treated with carcinogens, it nevertheless does serve to indicate certain features, more especially the pronounced depilatory effect of the first four carcinogens, in the early weeks. The figures under the names of the compounds indicate the initial number of mice in the corresponding experiment. The highest points (*e.g.* at the right hand end of the methylcholanthrene curve) represent a value of 100 per cent.

distinction is clearest in the early weeks, i.e. during the first phase of hair loss and before the complications introduced by alternate hair loss and regeneration have started to operate. But over the 18-week period as a whole it can be seen that hair loss was in general greater with the four potent carcinogens than with any of the other substances. On the other hand it is clear that 7-methyl-1,2-dibenzanthracene has a more pronounced effect than the weak carcinogens 1-benzophenanthrene and 1,2,5,6-dibenzacridine which appear to be about equal to each other and

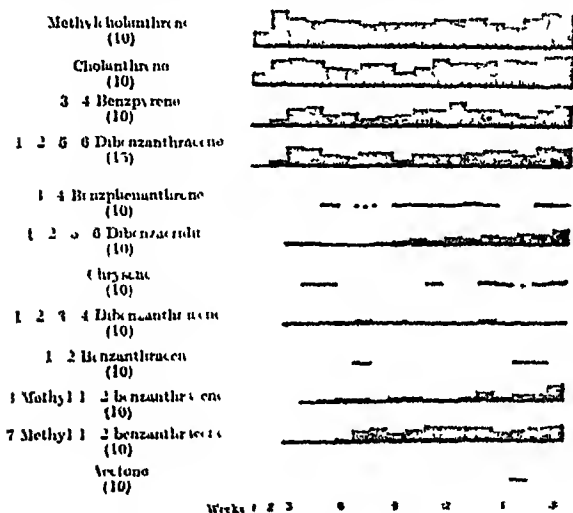


FIG. 2—Depilatory effect of various carcinogens on guinea pig skin (in acetone).

Explanation of symbols: (1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11) (12) (13) (14) (15) (16) (17) (18)

to 3-methyl-1,2-benzanthracene in depilatory power. It should be noted that the high late value with 1,9-dimethyl-1,2,3,4-tetrahydronaphthalene is largely attributable to an unusually high mortality rate of mice.

The cycles of epilation and hair regeneration are an important aspect of the process. It would not be entitled to regard the process as a purely destructive one but rather as a cyclical process, similar to the normal cycles of anagen and catagen.

period of five months' treatment with a potent carcinogen, each mouse passes through several such cycles. This is not the case in the hair loss seen with some irritants in which the main reaction is destructive and inflammatory, and the hair loss is only slowly made good.

It is seen that a general statement cannot be made that epilation is characteristic of carcinogenic hydrocarbons as opposed to their non-carcinogenic analogues. But it appears to be true that, for the six carcinogens tested, the depilatory phenomena take place at rates and magnitudes in the same order as those of carcinogenesis, *i.e.* methyl cholanthrene = cholanthrene > 3 4-benzopyrene > 1 2 5 6-dibenzanthracene > 3 4-benzphenanthrene = 1 2 5 6-dibenzacridine.

**Other macroscopic changes** The surface, which has a matt appearance at the time of the first alopecia, changes in appearance from week to week, becoming rather rough and uneven as the time for tumour formation approaches. Ulceration is not infrequent, but does not appear to be a necessary precursor of tumour formation. Indeed, the impression obtains that ulceration as such is an antagonistic process to carcinogenesis, as many tumours have been seen to arise in animals which never displayed ulceration, and in the others tumour formation only occurred when healing of the ulcers was complete or nearly so. Tumours at their first appearance were not related to ulcers, but arose in different parts of the skin. It is believed that ulceration is not in itself a direct result of the action of carcinogenic hydrocarbons, but rather a secondary outcome from the diminished resistance to injury and infection brought about by interference with the normal structure, *e.g.* the integrity of the fur. Some of the non-carcinogenic hydrocarbons, and also pure benzene, produced ulceration at times in mice which were deficient in hair, and the two phenomena seemed to be closely related. Thus, with chrysene dissolved in benzene, at one stage about half of the mice were seriously deficient in hair and also ulcerated, while the other half showed almost normal fur and no ulceration. Ulceration was significantly less in all cases where acetone solutions were used.

During the course of an experiment with a carcinogen, there is progressive thickening of the skin. In the early stages this is only just perceptible to the touch and suggests the presence of oedema, later it becomes more apparent, as there is an addition to the connective tissue of the dermis. It develops most rapidly and is most marked with the potent carcinogens. Associated with it there is a progressive loss of elasticity, especially well shown during excision of the skin immediately after killing the mouse for histological examination. The normal skin curls up at the edges, so that it has to be manipulated before it will lie flat on the filter



paper After some weeks' treatment with a potent carcinogen the thickened skin on removal remains flat, so that it is much more readily handled

At the same time, local congestion becomes conspicuous On reflecting the skin of the back the treated area is more or less sharply defined on the deep surface by the congested vessels This phenomenon was extensively studied by Kroyberg (1929) in tared mice by intravenous injection of india ink, and has recently been confirmed by Hval (1937) for mice treated with 1 2 5 6 dibenzanthracene The congested vessels are very conspicuous, however because of their increased calibre and blood content, even without the injection of india ink or other material, though such procedures are of value in securing convincing permanent preparations The present writer believes that the congestion should be regarded as a passive and probably obstructive phenomenon, indicative of a functional ischaemia, this point will be discussed later For the present, it should be stated that most of the engorged vessels seemed to be veins

The last two findings, loss of elasticity and venous congestion, are phenomena which appear to be of importance in relation to carcinogenesis They have been observed occasionally with the non carcinogens especially the methyl benzanthracenes but even with the latter they only occur rarely when acetone solutions are used On the other hand, they may be regarded as constant changes with the four most potent carcinogens and there is a suggestion that the same would have been true for 1 2 3 6 dibenzanthracene and 3 4-benzphenanthrene if a sufficiently large number of animals had been treated with these substances for an adequate time Furthermore, the shorter the average tumour induction time of a carcinogen, the more rapidly does it produce congestion and non elasticity of the skin

The formation of tumours may be passed over briefly as there were no differences observed between these tumours and those produced by tar The latter have already been very thoroughly described in the literature Two points, however seem to be worth mentioning The earliest warts produced by pure hydrocarbons grow slowly and not infrequently regress, when they persist they often take a considerable time to reach the stage of infiltrative cancer, while many are still "benign" at the death of the animal Malignancy seems to develop more rapidly in those nodules which appear later in an experiment The similarity to tar is obvious The second point is that the rate of growth of the tumours does not seem to be related to their rate of production by different hydrocarbons The present material is insufficient for statistical analysis, but allowing for the wide variations in growth rate of the tumours produced by any carcinogen, it does not appear that

tumours produced by, say, methylcholanthrene enlarge any more quickly or slowly than those produced by dibenzanthracene or tar. This is as might be expected, since there is no reason to believe that the carcinogenic hydrocarbons play any part in the growth as such of the tumours induced by them. It is especially difficult to see, for instance, how they could affect the growth of such a tumour after multiple transplantations. There is, indeed, evidence that they may actually inhibit the growth of neoplasms (Haddow and Robinson).

### *Histological changes*

The material available consisted of 237 mice treated with 1, 2, 5, 6-dibenzanthracene (of which 144 had been subjected to additional experimental procedures), 141 mice treated with 3, 4-benzpyrene, 100 with methylcholanthrene, and groups averaging 20 to 25 with each of the other hydrocarbons in the list given previously. The larger numbers in the case of the first three compounds are due to the fact that histological preparations existed derived from previous experiments, in these benzene had been used as the solvent, and while benzene itself produces changes in the skin, it is still possible in this material to confirm many of the findings. Much sharper and more reliable results are obtained with acetone solutions, however, as applications of acetone itself have been found to be almost completely inert over the experimental period. It will be most convenient to deal with methylcholanthrene first, and then to indicate differences in the action of the other compounds.

**Effect of methylcholanthrene** It is to be understood that where times are given, they are to be regarded as averages. The rate at which changes develop varies, as might be expected, between one animal and another.

At the end of the first week, *i.e.* after a single application of methylcholanthrene, definite changes are already present (fig. 3). The epidermis, which in the normal mouse consists of two or three layers of small cuboidal cells, is already hyperplastic and in its stratified structure bears more resemblance to human than to normal mouse skin. The epithelium is many layers thick, the cells have assumed a typical squamous appearance, and it is possible to distinguish a stratum malpighi with prickle cells, a well marked stratum granulosum, and a considerable superficial keratinous layer. The connective tissue of the dermis is normal. The subcutis (by which is understood the adipose layer between the collagenous dermis and the panniculus carnosus) is congested, but free from cellular infiltration. The hair follicles are strikingly altered, as might be suspected from the depilatory phenomena. Most of them are represented by short club-like solid downgrowths of cells



PLATE LI

FIG 3 —Skin of mouse 1 week after methylcholanthrene 0.3 per cent in acetone. Hypoplasia and keratinisation of epidermis, hair follicles shrunken and devoid of hairs.  $\times 35$

FIG 4 —Three weeks methylcholanthrene in acetone. Active regenerative changes in hair follicles and bulbs.  $\times 35$

FIG 5 —Three weeks methylcholanthrene in acetone, to show commencing change in dermis. In the superficial dermis the relatively coarse normal collagen has been replaced by finely fibrillar collagen. The alteration in the collagen is most conspicuous at the extreme left, where the entire thickness of the dermis is changed. Note also absence of cellular infiltration.  $\times 85$

PRECANCEROUS SKIN CHANOLS



FIG 3



FIG 4



FIG 5



attached to the base of the epidermis and only penetrating a short distance into the dermis. It is impossible to make out the distinction between the different cell layers of the normal follicle and the impression created is that with the loss of the hair there has been a loss of the internal root sheath, accompanied by an upward shrinkage of the external root sheath. When the mouths of the follicles are still detectable, they are generally blocked with keratinous plugs.

This epidermal increase reaches its maximum in the first week and thereafter shows little difference, quantitative or qualitative until immediately before tumours are going to make their appearance. After two weeks there is still no significant change in the connective tissues of the dermis, and the subcutis is as before. The hair follicles, however, may show a different picture, depending on whether regeneration of hair has started or not. If active regrowth of hair is taking place the hair follicles are greatly enlarged and sometimes dilated, the colls of the internal root sheath are unusually prominent as a result of their increased size and intense eosinophilic staining, the external sheaths are also hyperplastic, the bulbs are now large and composed of crowded, darkly staining cells (normally in the resting skin of the mouse only occasional bulbs are seen) and they penetrate deeply into the subcutis (fig. 4).

In three weeks changes are seen in the dermis. Normally there is no differentiation in the mouse, as there is in some species into two layers of different texture, the whole dermis consists of interwoven, rather coarse bands of highly refractive collagen fibres (cf. fig. 14). After three weekly applications of methylcholanthrene the superficial parts of the dermis are altered in texture (fig. 5). The collagen fibres are fine and have lost their refractivity. The amount of dermis involved is variable: sometimes only a narrow subepithelial zone is affected, sometimes the process penetrates quite deeply. It is not clear whether the phenomenon represents an alteration in the fibrillary texture of the pre-existing collagen, or whether the fine fibrils should be regarded as a new formation. The former interpretation is preferred, both on account of the rapidity with which the process appears and because the total thickness of the dermis at points where it is marked is not greater than at places where there is relatively little change. About the same time mast cells begin to increase in number in both dermis and subcutis. The epidermis remains in much the same condition as previously, except that the superficial keratinous layer is perhaps somewhat denser in structure. The hair follicles are mostly in the active hypertrophied condition.

The first detectable changes in the elastic tissue are found after four or five weeks. There is a gradual loss of elastic fibres in the superficial part of the dermis. Normally, the elastic fibres of mouse

dermis continue up to the epidermal margin, are most numerous in the superficial half of the dermis and most prominent around the necks of the hair follicles. There is not at this time any alteration in the texture of individual elastic fibres, only a gradual disappearance of the superficial fibres leaving small subepithelial zones of elastic-free dermis. The alteration in the collagen is becoming progressively more marked and in some places the whole thickness of the dermis may have assumed a rarefied appearance due to the replacement of the normal by the fine, non-refractile fibres. The fact that the disappearance of the elastic lags behind this process seems to be additional evidence against the fine collagen being a new formation.

From this time onwards till the appearance of warts (generally between the tenth and sixteenth weeks) there is a progressive increase in the various changes described above. Of these the most important seems to be the change in the character of the dermal collagen. This goes on until in many cases the entire dermis is transformed into the rarefied type (fig 6). Later, further changes may occur, such as hyalinisation of the collagen, or the re-formation of coarser fibres which, however, are straight and fail to show the intertwining characteristic of the normal, or the dermis may retain its "thin," rarefied appearance. As time advances there is sometimes an increase in thickness of the dermis, suggesting that in the later stages at any rate there is a new formation of collagen.

This new formation of collagen extends in places to the subcutis, so that scars are formed, and it has been found that the site of appearance of tumours was related to these scars in a significantly high proportion of cases (*cf* figs 10 and 11).

The further changes in the elastic tissue are somewhat variable. At first there is always a decrease in total elastic, due to its removal from the superficial dermis. As time proceeds, in some mice the elastic tissue remains subnormal in amount, in others (a majority) there is an increase. The new elastic tissue is different in texture from the normal, slender, gently curved fibres. It is seen as tangled masses of fibres or closely packed fragments of granular elastic (fig 7). The elastic hyperplasia is patchy and frequent elastic-free gaps are seen in the dermis. Very often tumours make their first appearance at the site of such gaps (fig 8). It is not, of course, easy to be certain in such cases whether the tumour is subsequent or antecedent to the elastic defect, but it may be said that such defects have been frequently seen in mice killed immediately on first detecting a wart. They have also been seen underneath areas detected microscopically and believed to be early stages in papilloma formation.

With the exception of mast cells, which become very numerous







## TUMOROUS CHANGES



FIG. 8.—Papilloma after 12 weeks of treatment with 3% benzpyrene in benzene. Stained with elantherin to show gaps (black) in elastic tissue beneath papilla. In previous section note infiltration of mast cells with the elastic tissue.



FIG. 9.—Twenty one weeks 3% benzpyrene in benzene. Beneath the papilla is a small subcuticular scar, also more extensive fibrosis of fat and beneath an area of irregular epithelial hyperplasia which may be an early stage of neoplasia.  $\times 10$ .

confirmatory evidence for the view that the subcuticular scars are genuinely antecedent to the papillomata with which they are associated, in that they are more marked than is apparently to be expected as the result of a secondary reaction to neoplastic proliferation of squamous epithelium

The carcinomata which have been seen in the present series have varied from well differentiated squamous and keratinised carcinomata to highly anaplastic spindle-celled tumours, some of them simulating leiomyosarcoma

**Effects of other carcinogenic hydrocarbons.** *Cholanthrene* is practically identical in its behaviour with methylcholanthrene. It happens, however, that the earliest tumour of the present series occurred in a mouse which had received four weekly applications of 0.3 per cent cholanthrene in acetone. The mouse was killed immediately, and microscopically the entire dermis beneath the tumour (a rather large flat one) showed the altered type of collagen. Further, the subcutis showed fibrous scars, and the elastic was absent beneath the tumour and granular at its edges

3. 4-benzpyrene in 1 per cent solution produces the same effects at about the same rate as 0.3 per cent methylcholanthrene. In 0.3 per cent solution the effects are produced somewhat more slowly (fig 9)

1. 2. 5. 6-dibenzanthracene produces the same changes as methylcholanthrene, but at about half the rate (fig 10)

3. 4-benzphenanthrene and 1. 2. 5. 6-dibenzacridine gave somewhat equivocal results. In benzene solution, their effects may be regarded as qualitatively similar to those of the other four carcinogens though they progress considerably more slowly. In acetone solution, however, results have been obtained which necessitate great caution in interpretation. In some mice changes were negligible in periods up to 23 weeks, while other animals killed during the same interval showed changes of a kind which might be expected by analogy from the other carcinogens. In the acetone series one tumour was obtained with each of these substances. In both cases practically no changes were present in the skin except under the papillomata. In this situation the dermis was rarefied, the elastic broken and the subcutis fibrous (fig 11). The changes therefore correspond with those found under papillomata produced by more active carcinogens. On the other hand, the absence of changes in the rest of the treated skin should not be disregarded. The period of the experiment was not of course long enough to expect many tumours with compounds which take a long time to produce them. At the end of the experiment, therefore, two mice were allowed to survive and applications were continued. One of these developed a tumour after 28 weeks' treatment with 1. 2. 5. 6-dibenzacridine. It was immediately killed. Beneath the tumour

## PRINCINCLOSIS SKIN CHANGES



FIG. 8—Papilloma after 12 weeks of treatment, stained to show elastic tissue (between arrows) in elastic tissue beneath the papilloma. In this and previous section note intense staining of mast cells with the elastic tissue.  $\times 50$



FIG. 9—Twenty-one weeks 3.4 benzpyrene in benzene. Beneath the papilloma is a small subcuticular nodule also more extensive fibrosis of subcutis beneath an area of irregular epithelial hyperplasia which may be an early stage of neoplasia.  $\times 10$





times in the enlarged regenerative phase, sometimes in the resting normal phase, and after 23 weeks are still abundant. The superficial epithelium remains in substantially the same condition as when hyperplasia was first seen; indeed it has been seen at times to revert to the appearance of normal mouse epidermis.

In the case of solutions of the non-carcinogenic hydrocarbons in benzene, no changes are detected additional to those which might be expected with benzene alone, with three exceptions amongst the compounds studied, and omitting a few isolated observations. It is only necessary therefore to refer to these exceptions.

*7-methyl-1, 2-benzanthracene* in benzene gives epithelial hyperplasia within 2 weeks. The changes in the dermis begin a week later, and are variable. Sometimes they are not distinguishable from those produced by pure benzene, but in a considerable proportion of mice further changes of a rather active type are found. These seem to have a considerable inflammatory element and comprise oedema of the dermis, especially the superficial dermis, associated with leucocytic infiltration. The rarefied superficial dermis is frequently occupied by many large active fibroblasts. At certain stages the changes under low magnification resemble those produced by methylcholanthrene, but they differ in the presence of oedema and in the marked subepithelial fibroblastic proliferation. Further, the reaction is not similarly progressive and even after 23 weeks there may be a well marked deep layer of normal dermal collagen. Ulceration is more likely to occur with *7-methyl-1, 2-benzanthracene* than with the other hydrocarbons, and such subcuticular scars as occurred were attributed to this or to infection.

*3-methyl-1, 2-benzanthracene* in benzene produces reactions similar to those of the *7-methyl* derivative, but of less intensity.

A proportion of the mice treated with *chrysene* in benzene showed ulceration at an early stage of the experiment (6-8 weeks). Histologically the changes associated with healing of these ulcers were added to the benzene effects. At later stages of the experiment the *chrysene* mice showed no greater changes than those treated with pure benzene.

A single mouse killed after 14 weeks' treatment with *pyrene* in benzene showed a quite unusual hyperplasia of the elastic of the dermis, taking the form of a great increase in very fine fibres running in all directions. This appearance was not seen in any other animal, including the remainder of the *pyrene* series.

There was occasional evidence of elastic hyperplasia and granular elastic tissue in mice treated with *1, 2-benzanthracene* and *phenanthrene* in benzene (fig. 13).

Acetone solutions gave much more clear-cut results. It has already been stated that after 23 weekly applications of acetone itself the skin is still quite normal.





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PREGNANT UTERINE CHANGES

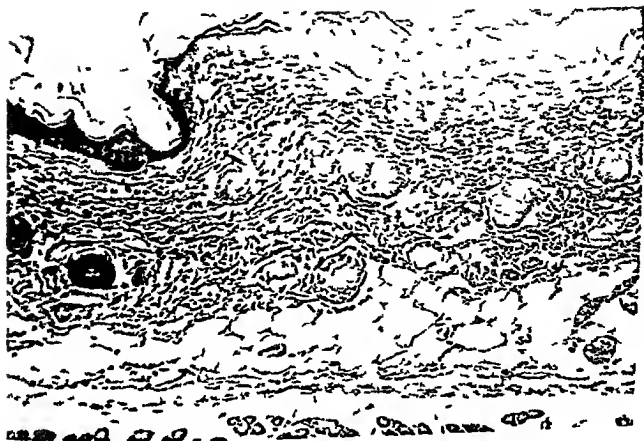


FIG 10



FIG 11



FIG 12

#### PLATE LIV

- FIG 10—Three weeks 1 2 5 6-dibenzanthracene in benzene. Already there are well marked epithelial hyperplasia and replacement of superficial dermis by fine-fibred collagen. The latter change is unusually advanced for this compound.  $\times 145$
- FIG 11—Eighteen weeks 1 2 5 6-dibenzacridine in acetone. Beneath the papilloma the dermis is altered and there is some subcuticular scarring. At the sides of the section the dermis is practically normal.  $\times 45$
- FIG 12—Benzene alone, 21 weeks. Most of the dermis is of normal texture except for a narrow subepithelial layer. Regenerative changes in hair follicles.  $\times 50$ .

With every non carcinogenic hydrocarbon tested some normal mice were obtained at the end of the experiment. This is significant, in that it tends to show that when changes did occur, additional factors must have played a part, whether they were intrinsic in the particular animal or due to external causes.

In two mice treated with 1,2-benzanthracene in acetone there was slight rarefaction of the superficial dermis after 18 and 21 weeks. The changes corresponded with those seen after 3 weeks with methylcholanthrene.

7-methyl-1,2-benzanthracene in acetone gave epithelial hyperplasia in one mouse after 3 weeks without changes in the dermis. Of 7 mice killed at 21 weeks, 2 were normal (fig. 14), 3 showed trivial changes and 2 showed extensive changes associated with inflammatory cell infiltration and oedema (fig. 15). It is therefore clear that the reaction to 7-methyl-1,2-benzanthracene in either solvent is not constant, but there does appear to be evidence that when inflammation is present this hydrocarbon intensifies its effects.

3-methyl-1,2-benzanthracene in acetone showed discontinuous epithelial hyperplasia in a mouse killed at 5 weeks. At later stages mice showed ulceration and leucocytic and lymphocytic infiltration of the dermis and subcutis. At 21 weeks one mouse showed inflammation and oedema similar to but less marked than with the 7-methyl compound in 2 mice at this stage. Other mice killed at this time were normal.

#### *Additional observations*

The results with non-carcinogenic substances seemed to show that certain changes brought about by carcinogens in the connective tissues of the dermis and subcutis are significant. The question arose as to whether any or all of these changes might be a secondary result of the epilation associated with the action of carcinogenic agents. To test this point, a group of mice were clipped in the interscapular region, and removal of the hair was continued when necessary over a period of some weeks, during which the mice were killed at intervals. Histological examination of their skins showed that at no stage did changes occur either in the epidermis or in other parts of the skin. The interesting observation was made, however, that the hair of these mice grew very rapidly at intervals, and very slowly in the intervening periods. During periods of active growth the histological appearance of the follicles is identical with that seen during the phase of regeneration after epilation by carcinogens, i.e. they were greatly enlarged and dilated, with an increase in the size and clarity of the cells of the internal root sheath, and enlarged hyperchromatic bulbs penetrating deeply into the subcutis. Thus

confirms the view that the occurrence of similar changes during the application of hydrocarbons represents a purely regenerative process and has no element in itself of a degenerative phenomenon

Further information corroborating the view that the changes in the dermis and subcutis are of importance in relation to tumour formation was obtained by studying the reactions to benzpyrene of two pure strains of mice bred in this laboratory by Mrs G. M. Bonser. There were available 19 mice of a strain in which tar tumours arise unusually early, and 25 of a strain in which the first appearance of tumours is much later than the average. Weekly applications of a 1 per cent solution of 3.4-benzpyrene in benzene were made and the mice killed at intervals for histological examination. It was found that the changes in the dermis, especially the replacement of the normal collagen by the fine-fibred non-refractile type, advanced more rapidly in the mice of the early tumour strain, and that at the time tumours appeared the dermal changes had reached approximately the same stage in each group of mice. It was also observed that the first epilation occurred more rapidly in the early tumour strain, the animals of which were all completely epilated after one week, regeneration of hair was also brisker in this group.

In order to compare the effects of the carcinogenic hydrocarbons with those of other chronic irritants, a study has been made of the skins of mice undergoing weekly treatment with the following applications:—20 per cent. formalin (= 8 per cent formaldehyde) in acetone, 10 per cent acetic acid in acetone, 10 per cent croton oil in benzene, 0.1 per cent mustard gas in benzene and in acetone and 0.06 and 0.03 per cent. of the same in liquid paraffin, 0.25 per cent cantharidin in acetone, 2 per cent iodo-acetic acid in benzene, 50 per cent turpentine in acetone and 20 per cent pyrogallol in acetone.

Formalin and acetic acid, in the concentrations used, are without histological effect on the skin. Iodo-acetic acid gave no changes which could not be attributed to the solvent benzene. Croton oil is qualitatively not unlike the carcinogens in its effects, but very much slower in altering the connective tissue, in periods up to 25 weeks there was always a well defined deep layer of normal dermis, it is possible that this substance is weakly carcinogenic, as a small papilloma occurred in one mouse. Mustard gas and cantharidin are similar in their effects. There is a considerable inflammatory element in the reaction, the dermis and subcutis are infiltrated with leucocytes and polyblasts and the superficial dermis contains many large active fibroblasts and is frequently cedematous (fig 16). Though macroscopic blistering is not observed in the mouse, microscopically the hyperplastic epidermis is often split into layers or separated from the underlying dermis by cedema fluid, and hydropic degeneration of the epithelial cells is common. Even at the end

## PRECARCINOUS SKIN CHANGES



FIG 13—Sixteen weeks  
there are gaps in

one in benzene. The elastic tissue is granular and  
is more frequent with carcinogens.  $\times 95$

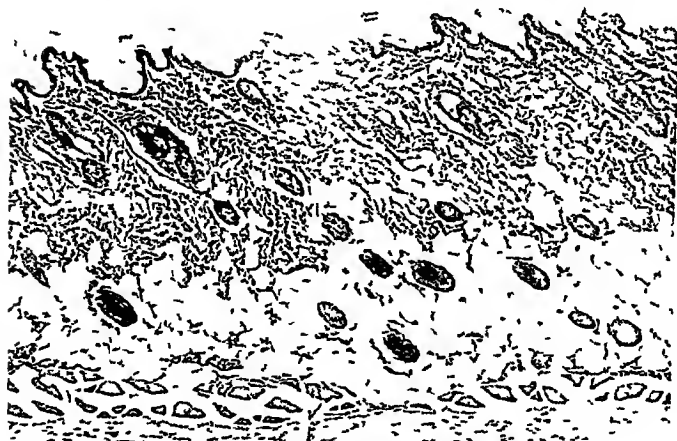


FIG 14—Twenty one weeks 7 methyl 1 2 benzanthracene in acetone. Skin quite normal  
hair follicles in a normal active phase.  $\times 95$





PRECANCEROUS SKIN CHANGES



FIG 15—Eighteen weeks 1-methylanthracene in benzene. Dermis is altered but change is accompanied by leukocytic infiltration and edema, best seen in the superficial dermis.  $\times 70$

1-methylanthracene in benzene. Dermis is altered but change is accompanied by leukocytic infiltration and edema, best seen in the superficial dermis.



FIG 16—Nineteen weeks cantharidin in acetone. Changes very similar to those of fig 15.  $\times 70$



of 28 weeks there is generally an intact deep layer of normal dermis, and subcuticular scarring does not occur in the absence of ulceration. Of the substances studied, turpentine approaches most closely in its effects to the carcinogens, but again inflammation is a more conspicuous feature, as shown by infiltration with leucocytes and polyblasts. As compared with the carcinogens, the epithelial changes are disproportionately greater than the dermal, turpentine always leads to loss of hair, but not to complete alopecia at any stage. Pyrogallol gave a somewhat peculiar result in that epithelial hyperplasia developed slowly over many weeks, accompanied by a slow but progressive epilation in which recovery phases did not occur. The changes in the collagen of the dermis were trivial unless infection or ulceration took place.

### DISCUSSION

Comparison of the present findings with those obtained in earlier experiments with coal-tar shows somewhat unexpectedly that the course of the reaction with the latter substance has many points of similarity to that with the pure carcinogenic hydrocarbons. In the early stages of tarring the similarity is not so apparent, as there is always a considerable inflammatory reaction, with infiltration by leucocytes and other cells. It is reasonable to suggest that these acute inflammatory phenomena are brought about by constituents of tar other than the carcinogenic component benzpyrene. In later phases of tarring the inflammatory changes subside, and the histological picture becomes much the same as that seen with the most active of the pure carcinogenic hydrocarbons. Moreover, the cyclic epilation and regeneration of hair is also a feature of tumour induction by coal-tar.

While most of the old histological work on tar cancer is concerned with the epithelial changes, a number of authors directed attention to the possible importance of changes in the deeper tissues. Döderlein (1926), for example, found changes in the collagen of the dermis after 14 days, and after three months there was much granulation tissue in the dermis and the superficial layers of elastic tissue were broken up. His view was that the dermal changes affected the nutrition of the epithelium, so that there gradually emerged an aggressive type of cell (the cancer cell), the product of its difficult life conditions. Guldberg (1931) points out that almost from the beginning there is an increase in the finely fibrillar collagen of the dermis, advancing as the experiment proceeds, and inconspicuously accompanied by changes in the elastic tissue. He confirms the work of Kreyborg on the importance of congestion and the formation of telangiectases. Borgh (1926) found that in the tared ear of the rabbit the connective tissue fibres showed a process of dissociation which preceded deep proliferation of the epithelium.

In comparing the effects of pure chemical carcinogens with other applications, the chief changes which appear to be of significance are (i) the alteration of the collagen of the dermis to a fine-fibred, non-refractile type without associated cellular activity, (ii) alterations in the structure rather than the quantity of the elastic tissue, (iii) congestion of the subcutis. In respect of the actual sites at which tumours arise, fibrosis of the subcutis and gaps in the elastic of the dermis may be significant, though it is important to remember that these points can only be adequately proved for papillomata; their relationship to carcinoma must remain *sub judice*.

Additional changes which occur with carcinogenic substances may be duplicated by other (non-carcinogenic) applications. Squamous hyperplasia of the epidermis is seen, for instance, with 7- or 3-methyl-1,2-benzanthracene in a proportion of mice, and with turpentine, cantharidin, etc. and it will develop more slowly with benzene alone. Mast cells are always conspicuously increased with the carcinogens, but the same applies to all substances which produce a reaction of any kind in the skin. The hair follicle changes with carcinogens, but all these can be produced by non-carcinogenic substances, with the exception of the final change in the cases where hair follicles take part in tumour formation.

An interesting aspect of the carcinogenic reaction is its progressive, non-inflammatory character. Leucocytic infiltration of the tissues plays no necessary part in the reaction. That it so occurs may be attributed to accessory circumstances such as in the case of turpentine. With the great majority of the other substances tested an inflammatory factor is present in the changes produced. Indeed, it is strong evidence that in the absence of other factors none of the non-carcinogenic hydrocarbons produce any changes whatever when dissolved in acetone.

The results obtained with 1,2:3,6-dibenzacridine and 1,2:3,6-benzfluoranthrene are difficult to interpret. Undoubtedly it is possible to get almost normal skins after many weeks' treatment with these compounds. On the other hand, when the skin is present the underlying tissues always show the changes which have been postulated above as characteristic of the carcinogenic reaction. It may be that these weak carcinogens, requiring a long time to produce tumours, operate most effectively on the skin in which for some reason conditions are favourable to them.

The present results tend to show that the action of some hydrocarbons is in a sense an indirect one, in that the change in the epithelial cells may be a secondary result of their environment arising from the reactions which take place. That there is a primary effect on the cells themselves is a certain in view of the early changes.

which they exhibit. But this early hyperplasia is also obtained in a histologically identical form with substances which are never carcinogenic. This epithelial increase in the case of the most potent carcinogens is almost immediate, and attains its full extent within the first week, after which there is but little change until the time, about three months later, when tumours are going to arise. Two explanations are possible. First, after the primary hyperplasia has been achieved, the action of the carcinogen may enter upon an entirely different phase, so that the cells are progressively altered by its action to tumour cells without undergoing further numerical increase. Alternatively, the direct action on the epithelium may remain similar throughout the process, surface loss of cells counterbalancing hyperplasia after a certain point has been reached, and its cancerisation be a secondary result of changes in the deeper tissues. To the author, the second hypothesis seems the more tenable for various reasons of which two may be mentioned. There is no great difference in the times taken to produce epithelial hyperplasia by methylcholanthrene and 1, 2, 5, 6 dibenzanthracene, but the rate of progress of the changes in the dermis is much slower with the latter substance, and the average time taken to induce tumours with it is correspondingly longer. The macroscopic character of "precancerous" mouse skin is very typical; it is not only thickened, but has lost its normal elasticity, such changes seem to be more appropriately referred to the dermis than to the epidermis.

The changes in the elastic tissue itself have already been mentioned. They are of interest in relation to the work of Bierich (1927) with tar. He found that the elastic was increased constantly in tarred mice, and he attributed this to an over production of lactic acid. Many subsequent authors have disagreed with this statement, and have found that while increase in elastic may occur in tarred mice, the phenomenon is by no means constant. The present work with pure hydrocarbons gives similar results, the amount of elastic is frequently increased, but by no means invariably. It has been found markedly increased with non carcinogens. The alteration in the character of the elastic tissue appears a more significant phenomenon.

Evidence has been given previously (Orr, 1934, 1935, 1937) that the origin of tumours may be to some extent attributed to functional ischaemia of the sites at which they arise. There is an indication of marked vascular alterations in the subcuticular congestion which is a feature of the action of carcinogenic hydrocarbons. Attention was directed to the vascular aspects of tar carcinogenesis by Kreyberg, who believed that the congested vessels indicated the presence of a true hyperemia with over-nutrition of the epithelium, the latter being responsible for its assuming neoplastic properties. Reasons have been given in the previous papers for

disagreeing with Kreyberg's view, and for regarding the congestion as passive. This attitude is shared by Hval, who applied Kreyberg's technique to the study of the action of 1, 2, 5, 6-dibenzanthracene. If it is true, the excess blood in the tissues is stagnant, and the effective blood supply reduced. This is in keeping with the considerable accumulation of evidence in other directions, *e.g.* metabolism, that tumours arise in cells which are suffering from oxygen lack. The subject is raised again because it seems possible that the fibrous scars which have been observed in the subcutis of the present mice may be of importance in the mechanism of interference with the local circulation.

### SUMMARY

An investigation has been made of the changes in the skin of white mice treated with six carcinogenic hydrocarbons, ten non-carcinogenic hydrocarbons of related chemical structure, and a group of unrelated irritants. They have been used in solution in benzene and acetone, the latter solvent being of greater value for the present purpose as it is without effect on normal mouse skin.

Early epilation is a striking feature of the action of cholanthrene, methylcholanthrene, 3, 4-benzpyrene and 1, 2, 4, 6-dibenzanthracene, but not of 1, 2, 5, 6-dibenzacridine and 3, 4-benzphenanthrene. It occurs with some non-carcinogenic hydrocarbons, but to a much less extent than with the four most potent carcinogens. After epilation by a carcinogen, the regenerated hair is abnormal in character.

The appearance of tumours is preceded by a progressive thickening and loss of elasticity and by passive congestion of the skin. Ulceration of the surface does not bear any relationship to tumour formation.

Histologically, the carcinogens produce squamous hyperplasia of the epidermis in the first weeks of treatment. Thereafter, the progressive changes are found in the deeper tissues and the most significant phenomena are —

- (1) Transformation of the collagen of the superficial, and later of the whole, dermis into a fine-fibred, non-refractile type
- (2) Passive congestion of the subcutis
- (3) Alterations in the texture of the elastic tissue, without necessarily any increase in its amount
- (4) Absence of inflammatory cell infiltration

When tumours appear, they are frequently related to fibrous scars in the subcutis, to gaps in the elastic tissue of the dermis, or to both.

In comparing the carcinogens, the rate of progress of the changes cited corresponds with the rapidity with which tumours are induced.

Similarly, mice in which tumours appear early or late are those in which these changes advance rapidly or slowly respectively

When comparable changes occur with non-carcinogenic applications, evidence of inflammation is usually present

It is suggested that the cancerisation of the epithelial cells by the carcinogenic hydrocarbons is at any rate partly the result of the changes in the deeper tissues, and that its mechanism may be related to the consequent interference with their nutrition

My very sincere thanks are due to Professor J W Cook, F R S, who supplied me with a selection of hydrocarbons which would not otherwise have been obtainable and without which this work would have been impossible, and to Professor E L Kennaway F R S, who discussed the results with me and offered many helpful comments

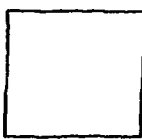
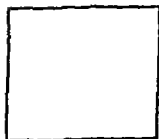
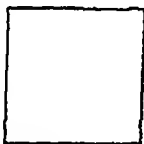
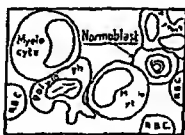
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# PLATE LVII



17

18

19

20

the cells are supravitaly stained, it is still quite marked even at 25° C. Motility will continue for hours provided that the coverslip is efficiently sealed, the preparation kept reasonably warm, and the light not used for long periods of time. Non-motile cells such as myeloblasts may show some changes in cell shape, as from round to oval. These changes appear to be produced by mechanical causes such as convection currents, pseudopodia and other signs of true motility are never seen.

*Cell outline and nucleus* The cell outline appears as a thin line of light. The outline of the nucleus is equally well demarcated and the central portions have a reticular structure.

*Granules* The most striking cytoplasmic structures are the granules found in mature granulocytes, they are large, round and brilliantly illuminated. The exceedingly large granules of eosinophils are resolved into rings of light (fig 3).

In supravitaly stained preparations other granular structures appear, particularly in primitive cells, such structures, because they stain with neutral red, are known as neutral red bodies. In unstained preparations all neutral red-staining structures appear as round granules which are illuminated in the same manner as the granules of mature granulocytes, they vary somewhat in size and the larger are resolved into rings of light. These neutral red granules can readily be identified by reason of their characteristic distribution in particular cells.

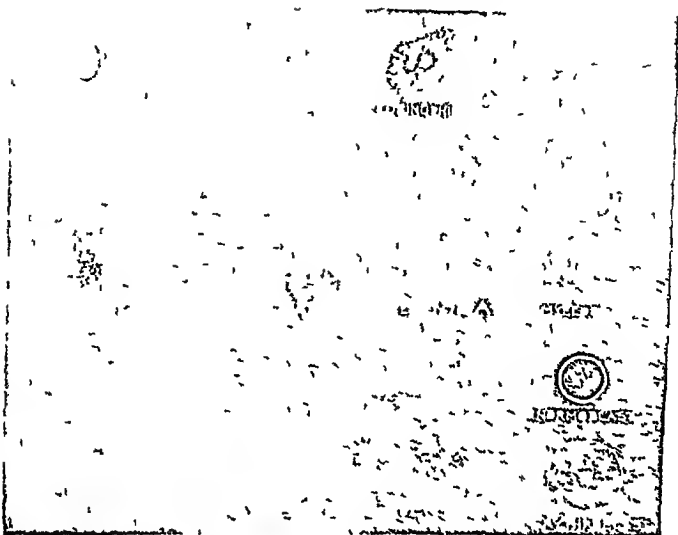
*Mitochondria* For some unknown optical or other reason, mitochondria are much less intensely illuminated than the granules. Mitochondria vary considerably in size, they may be fine dust-like points of light (fig 8) or thin elongated filamentous structures (fig 11), in certain cells, particularly lymphoblasts, they may be somewhat thicker and may even assume a round or oval form (fig 10).

#### *Individual cells*

*Red cells* The erythrocyte appears as a thick bright ring of light (figs. 1, 14-16 and 18-20), the cells clump together in masses and rouleaux, leaving clear spaces in which the leucocytes lie. Nucleated cells have the same thick cytoplasmic border, the dense nucleus of the normoblast and the cartwheel nucleus of the erythroblast are easily identified (figs 12, 13 and 15).

*Granular cells* The *neutrophil polymorphonuclear leucocyte* (figs 2, 14 and 15) is the most active cell seen. In shape it is elongated and irregular, in size some 15-20  $\mu$  long by 5-10  $\mu$  broad, and it moves across the field at a rate of about 30  $\mu$  per minute. The lobes of the nucleus are easily visible, they appear as clear areas with a faint outline and chromatic structure. The cytoplasm is filled with relatively coarse bright granules which constantly stream in sympathy with the motion of the cell. There is a very

GROUND ILLUMINATION



Diagrammatic representation

Leucocytes with dark ground illumination

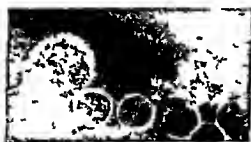


FIG 14—Motile and resting neutrophils

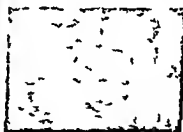


FIG 15—Two myelocytes two neutrophils and a normoblast



FIG 16—Myelocyte B



FIG 17—Myeloblasts



FIG 18—Large lymphocyte



FIG 19—Lymphoblast

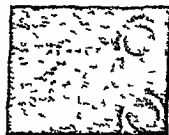
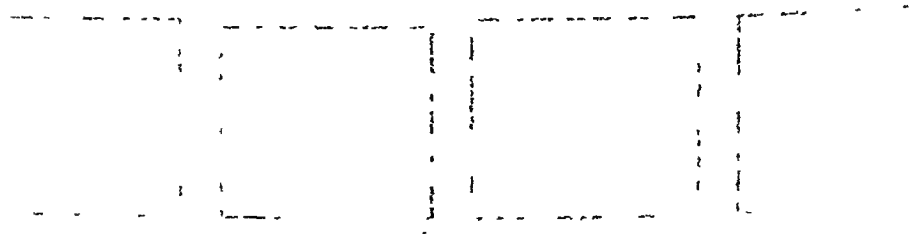
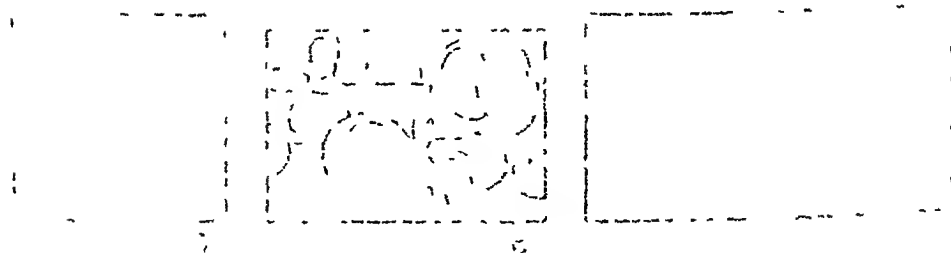
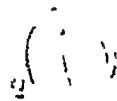
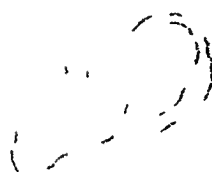
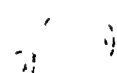
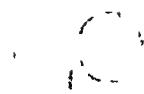
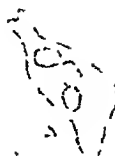
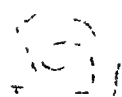


FIG 20—Monocyte

Dark ground photographs of living leucocytes. The definition is imperfect owing to depth of focus and movement of the cells. The luminosity of the cells is not comparable because of differences in exposure.









The *lymphoblast* (figs 10 and 19) is similar to the myeloblast, but the chromatin structure of the nucleus is denser whilst the mitochondria are larger and either oval or round. The mitochondria are either grouped round the nucleus or scattered diffusely. There are no granules. The cell is distinguished from the myeloblast by the much greater size of the mitochondria and their less close packing. Maturation of the cell is indicated by the appearance of bright granules, in acute leukaemia the number of these may greatly exceed that seen in the normal mature cell.

The *monocyte* (figs. 11 and 20) is a large amoeboid cell about  $7.15 \times 20 \mu$  in size. Two or more blunt pseudopodia are usually present. The shape of the cell constantly but slowly changes. The nucleus is commonly U-shaped, but may be circular or trefoil. Fine dust-like and thread-like mitochondria are scattered throughout the cytoplasm, with the coarser, brighter granules amongst them. The granules occasionally form a rosette.

The *premonocyte* is larger, more active, and more richly supplied with granules and mitochondria than the mature cell. The shape and motility of the premonocyte and the uniform distribution of its mitochondria and granules distinguish it from the myelocyte B.

### Discussion

Living preparations of blood examined by dark-ground illumination show features sufficiently characteristic to enable both primitive and mature leucocytes to be identified. We have noticed that cells which appear highly primitive in a Romanowsky-stained preparation are found, on examination either after supravital staining or unstained by dark-ground illumination, to be more mature than the stained dried film would lead one to suppose. It is extremely rare to find true myeloblasts in the peripheral blood, what appears to be a myeloblast in a Romanowsky film is almost always a myelocyte A in a vital preparation.

The lymphoblast is readily distinguished from the myeloblast both by its nuclear appearance and by differences in the size and distribution of the mitochondria.

A feature of the monocyte series is that the more primitive cell—premonocyte—is more motile than the mature cell. This is the reverse of what is found in the myeloid series and is not only a valuable differentiating point but one which may eventually throw some light on the origin and development of the monocyte.

Our thanks are due to Professor McIntosh for much helpful advice and criticism and to the Lady Tata Memorial Trust for a grant to one of us (M. H.).

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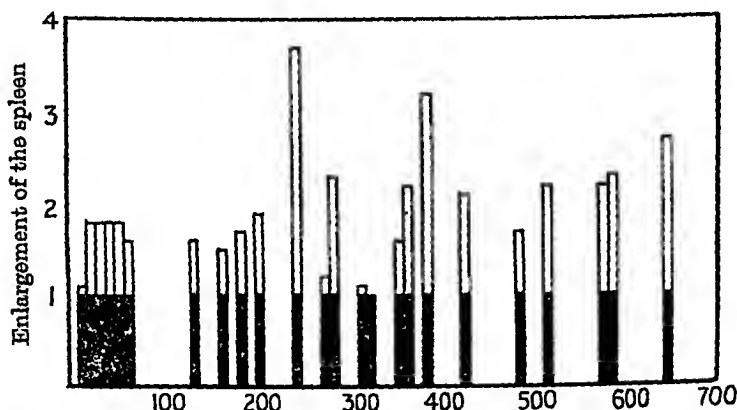




In 18 hours the spleen showed little macroscopic change. Histologically the syncytial nuclei of the pulp and the nuclei of the marginal zone of the malpighian follicles showed thickening and irregularity of the nuclear membrane and there was swelling of the cytoplasmic syncytium. The latter condition resembled cloudy swelling rather than simple oedema. In places, acidophilic staining was marked, indicating the beginning of coagulation necrosis. Occasionally frank peripheral necrosis of the follicular syncytium and of the lymphoid cells was present. The free cells in the pulp were not so much affected. In 48 hours the whole syncytium appeared eosinophilic and the meshwork of the pulp became indistinct. The necrotic changes had affected some of the nuclei of the pulp and many of the lymphocytes showed karyorrhexis.

*Repeated large (toxic) doses*

Following 12 to 58 injections of 15-20 and 20-30 mg of manganese chloride per kg body weight, amounting in all to 330-



TEXT FIG 1—Showing degree of splenic enlargement, as judged by the spleen weights, in manganese intoxication in rabbits, the black columns represent the estimated normal weight. The abscissae show the total amount of the drug administered (in mg per kg body weight).

990 mg, the changes met with were as follows. Macroscopically the spleen showed an increase in size and weight averaging about twice the normal (text fig 1). The capsule was tense, greyish purple and slightly more opaque than normal.

Microscopically the *spleen* showed changes in the malpighian follicles and an erythrophagocytic reaction in the pulp and sinuses. Capsular thickening and fibrosis were generally present, but only to a slight extent. Most of the malpighian follicles were affected (fig 1), but the change appeared more advanced in some than in others. In many cases coagulation necrosis of the cytoplasmic syncytium of the marginal zone of the follicle was followed by collapse of the mesh, and a gradual increase in the argentophile reticulum fibres could also be made out. In the later stages,







marginal zone from multiplication of the cells in this region. These dividing cells had pale oval nuclei and resembled germ centre cells. Regeneration seemed to commence in two zones, one at the centre of the follicle as in man, the other at the periphery where the change appeared more marked. Erythrophagocytic reactions were less marked both in pulp and sinuses. Sinus congestion and engorgement of the pulp veins in the chronic cases indicated that the cirrhotic changes in the liver had caused a certain degree of portal stasis. Frequently the sinuses appeared distended with mononuclear and polymorphonuclear leucocytes, indicating that inflammatory changes were also present. Often these cells were present in clusters in the pulp. On the whole proliferative changes were more marked in the follicles than in the pulp. In five rabbits in which the injections were discontinued for a period of six weeks to study the regenerative effects, the proliferative changes in the follicles reached an extraordinary degree (fig. 4).

The liver fibrosis was similar to but slighter than that seen with repeated larger doses.

**Summary.** 1 In acute manganese intoxication in rabbits there is diffuse or localised perivascular toxic necrosis in the spleen corresponding to the liver necrosis.

2 In chronic manganese intoxication repeated periarterial necrosis is followed by fibrillary overgrowth and in the later stages by a spread of fibroblastic tissue from the vessels so that areas of periarterial fibrosis are formed. These changes in the spleen correspond to the early stages of peripheral necrosis and periductal inflammation in the liver.

3 A splenomegaly of moderate degree is an accompaniment of these early pre-cirrhotic changes.

4. With very small doses of manganese and during recovery from the manganese intoxication there is marked proliferative enlargement of the spleen. Well marked hyperplastic reactions are then found in the malpighian follicles suggestive of the condition called "lymphoid reticulosis".

5 Exaggeration of the erythrophagocytic activity of the spleen with formation of numerous hæmatophages which actively ingest red cells produces a condition suggestive of splenic anaemia.

## 2 CARBON TETRACHLORIDE INTOXICATION.

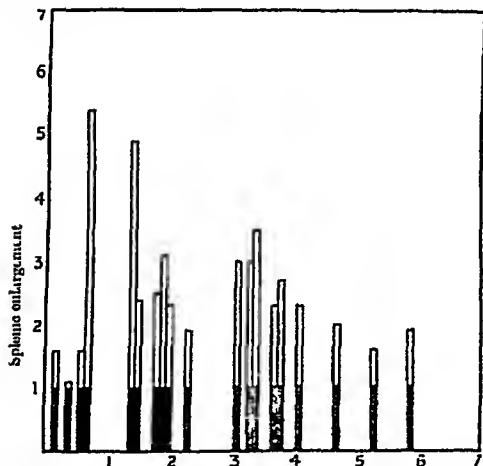
**Methods.** Thirty-two young albino rats together with 8 controls and 18 guinea pigs with 4 controls were used. The animals were kept under the same conditions and were fed on the same diet. Great care was taken to avoid any cage infection by cleaning and disinfection of the cages twice a week. For rats, pure undiluted carbon tetrachloride was given by means of a tuberculin syringe in doses ranging from 0.08 to 0.15 c.c. every third day, doses slightly less than those of Cameron and Karunaratne (1936). It was noticed that, at the commencement, doses larger than 0.1 c.c. caused

local necrosis at the site of injection but if the needle was introduced deeply into the subcutaneous tissue and the fluid spread out by massage, larger doses could be injected without much local damage. The injections were given into the skin of the abdominal wall near the middle line where the tissues are very lax and the skin is rather thin. Injections into the tail were invariably followed by necrosis. For guinea pigs, doses ranging from 0.1 to 0.25 c.c. were used but were so toxic as to cause death in 2-6 days. Hence the effect of repeated injections could not be determined in these animals.

Animals were killed by dislocation of the cervical spine and not by ether anaesthesia, as it was found that respiratory failure interfered with the vascular state of the spleen. The whole spleen and the splenic vessels, portions of the liver and the kidneys, heart and aortals were removed for histological examination.

### Results in the rat

The effect on the weight of the rat's spleen of various total amounts of carbon tetrachloride is shown in text fig. 2.



TEXT FIG. 2.—Showing the degree of splenic enlargement as judged by weight in carbon tetrachloride intoxication in rats. The black columns represent the estimated normal weight of the spleen for each animal (0.27 per cent of body weight). One case showing extreme hyperplasia to about six times the normal size is not included in this chart. The abscissa show the total amount of the drug administered (in c.c.).

### Single doses

After a dose of 0.5 c.c. carbon tetrachloride the spleen showed swelling of the syncytium, filling in of the pulp mesh and necrotic changes in the nuclei. Degenerative swelling of the pulp has been attributed to autolytic change (Perla and Marmorsten 1937),

marginal zone from multiplication of the cells in this region. These dividing cells had pale oval nuclei and resembled germ centre cells. Regeneration seemed to commence in two zones, one at the centre of the follicle as in man, the other at the periphery where the change appeared more marked. Erythrophagocytic reactions were less marked both in pulp and sinuses. Sinus congestion and engorgement of the pulp veins in the chronic cases indicated that the cirrhotic changes in the liver had caused a certain degree of portal stasis. Frequently the sinuses appeared distended with mononuclear and polymorphonuclear leucocytes, indicating that inflammatory changes were also present. Often these cells were present in clusters in the pulp. On the whole proliferative changes were more marked in the follicles than in the pulp. In five rabbits in which the injections were discontinued for a period of six weeks to study the regenerative effects, the proliferative changes in the follicles reached an extraordinary degree (fig 4).

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3. A splenomegaly of moderate degree is an accompaniment of these early pre-cirrhotic changes.

4 With very small doses of manganese and during recovery from the manganese intoxication there is marked proliferative enlargement of the spleen. Well marked hyperplastic reactions are then found in the malpighian follicles suggestive of the condition called 'lymphoid reticulosis.'

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owing to a spread of fibrous strands from the capsulo, but this was never marked. The malpighian follicles showed well marked hyperplastic reactions as in the earlier stages (fig 6). The cellular foci in the pulp were more diffuse and it seemed as if the reticulo-endothelial reaction had produced a free macrophage tissue in which numerous basophilic cells were present (fig 7). Between the pulp staining pulp nuclei, lymphocytes and basophilic cells and clusters of eosinophilic and neutrophilic leucocytes were present. Erythrophagocytosis was more marked than normal.

The liver showed well marked multilobular cirrhosis with strands of fibrous tissue breaking up the lobules. In many places the sinusoidal system had disappeared. Regenerative changes were marked.

#### Results in the guinea-pig

Carbon tetrachloride in doses of 0.15-0.20 cc caused death in two days in 8 animals. After two injections of 0.1 cc death occurred in two animals in 6 days. The effect of repeated small doses could not therefore be determined, even though Van Der Schueren (1932) claims to have produced cirrhosis with doses of 0.25 cc twice weekly for four weeks.

In animals dying after 48 hours the spleen showed slight necrotic reactions, chiefly in the pulp syncytium. Engorgement was not met with. Widespread cell accumulation was constant. Histio-cytic differentiation was quite marked in the pulp syncytium and littoral cell proliferation in the sinuses. Many free mononuclear cells thus appeared in the pulp, very often there were focal clusters, with numerous polymorphs and lymphocytes, both in the sinuses and around the vessels (fig 8). Clusters of basophilic cells similar to those met with in the rat's spleen were uncommon. Capsulo-trabecular changes were not distinct nor was pulp fibrosis met with. The cellular reaction appeared to be a primary response to the toxic agent, for there was no complicating sepsis and it was constant in all animals. In 6 days after injection the reaction had subsided.

**Summary** 1 Single doses of carbon tetrachloride produce diffuse syncytial necrosis and lymphorrhæxis in the rat's spleen. The necrotic reaction is occasionally localised to the marginal zone of the malpighian follicles. Guinea pigs show proliferative changes rather than necrosis. The liver at this stage shows acute necrosis.

2 With repeated administration of carbon tetrachloride to rats there is marked proliferation of reticulum in the spleen and fibrillary overgrowth. The liver shows collapse sclerosis.

3 Splenomegaly is often present in the early "pre-cirrhotic" stage.

4 With the development of hepatic cirrhosis there is super-

but here the grading of the reaction with different degrees of toxic damage indicates that the mesenchymal tissue of the spleen is just as liable to undergo toxic necrosis as parenchymatous cells

With a single dose of 0.2 c c the spleen showed a variable but diffuse congestion, slight lymphorrhaxis in the malpighian follicles, swelling of the pulp syncytium and opacity of the syncytial cytoplasm. Occasionally the nuclei appeared more opaque and pyknotic

### *Three doses of 0.2 c c every two days*

The spleen showed variable congestion. In the malpighian follicles hyperplasia of the marginal zone was suggested by the presence of pale-staining cells with oval nuclei possessing a thin chromatin meshwork. These resembled endotheloid cells derived from reticulum rather than lymphoblasts. Germ centres appeared in the centre of the follicles and the presence of numerous round cells with deep-staining opaque nuclei mostly confined to the central zone suggested lymphocyte formation. In places cells with basophilic cytoplasm and larger nuclei suggested that plasma cells were also being formed. Necrotic changes were not marked in the follicles. Small circumscribed areas of mononuclear cells and plasma cells, normally found in the pulp of the rat spleen, seemed to be undergoing active proliferation, these clusters were mainly grouped around the smaller arterial capillaries and the trabeculae. Necrotic changes in the syncytium occurred side by side with proliferative regeneration. The capsulo-trabecular system seemed rather thin and stretched and occasionally trabecular softening and necrosis could be made out, vascular changes were not definite.

### *Repeated small doses*

With 10 to 30 injections of 0.1-0.15 c c carbon tetrachloride, amounting in all to 1.8-3.3 c c, in two to ten weeks the reactions in the spleen were more or less similar (cf fig 5).

The liver now showed a condensation of the reticulum in the necrotic areas and a gradual spread of fibrous tissue into the collapsed areas indicating the commencement of active fibrosis. An irregular pattern of multilobular fibrosis was thus evolved.

With 30 to 52 injections of 0.08-0.15 c c of carbon tetrachloride given thrice and twice weekly, amounting in all to 3.4-5.9 c c, in 10 to 25 weeks the changes in the spleen were complicated by venous congestion caused by portal obstruction. This was shown by engorgement of the trabecular veins, pulp veins and sinuses, while the sinus congestion had also caused diffuse percolation of blood in the centre of the lobules. A proliferative growth of sinus tissue was not met with. Under the capsule, the sinuses were dilated and their walls appeared thin and more rigid than normal.

## CIRRHOTIC SPLEENOMEGALY

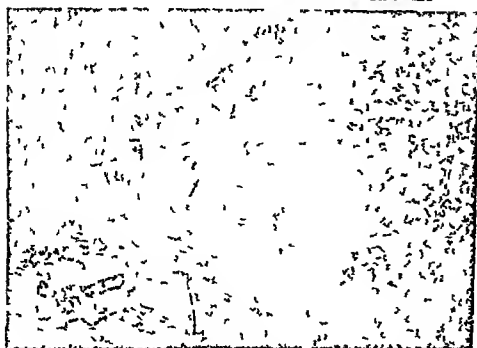


Fig. 4 — Rat spleen after 4 injections of 7.10 cc of carbon tetrachloride to a 1 kg body weight. One month after the injection extreme hyperplasia and regeneration of the malpighian follicles. H and E  $\times 120$

Fig. 5 — Rat spleen after 8 injections of 0.102 cc of carbon tetrachloride. Extensive necrosis of reticular synovium of pulp and of reticulum of malpighian follicles. H and E  $\times 175$

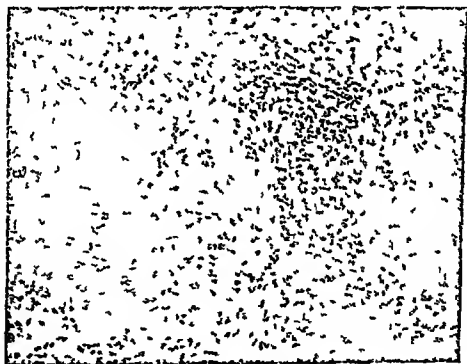


Fig. 6 — Rat spleen after 31 injections of 0.1 cc of carbon tetrachloride. Marked hyperplasia of malpighian follicles with widening of marginal zone (x) from proliferation of reticulum cells. o = the capillary mesh. H and E  $\times 120$

added congestive distension of the sinuses and of the pulp and trabecular veins

### 3 SENECEIONINE INTOXICATION

*Methods* Twenty albino rats averaging 170 g in weight were used. One g of senecionine was kindly supplied for the investigation by Dr J J Blackie, who had extracted it from *Senecio vulgaris*. This was dissolved in 100 c.c. of distilled water slightly acidified with acetic acid. Two to five mg were used for studying the effect of small doses and 10-20 mg for the production of acute lesions. The injections were given subcutaneously into the abdominal wall every third day. After the large doses the animals were killed when toxic jaundice was noticed, with smaller doses they were killed at fixed intervals.

#### *Large doses (10-20 mg).*

Forty-eight hours after the injection of 10-20 mg of senecionine the spleen showed a variable degree of diffuse congestion. The pulp syncytium had undergone extensive necrosis and because of its extreme swelling the mesh was almost indistinguishable. The free cells in the pulp were also affected and pyknosis of nuclei and diffuse lymphorrhesis were common. The necrotic changes were most marked around the malpighian follicles at the marginal zone. The liver showed the irregular blood "lagoons" described by Davidson (1935) with areas of necrosis between

#### *Repeated toxic doses (4-5 mg)*

Following two or three injections each of 4-5 mg of senecionine, the spleen showed well marked periarterial necrosis at the marginal zone of the malpighian follicles. Around the prefollicular arterioles and the arterial capillary ring separating the marginal from the central zones of the follicle the syncytial nuclei showed karyolysis and the cytoplasm had become condensed to form a narrow band of tissue. The change was so well marked that the malpighian follicles appeared bordered by well defined white rings of condensed cytoplasm (figs 9 and 10), in which the capillary lumen could not be demonstrated. Occasionally hæmorrhages were met with in these necrotic areas (fig 11). Even at an early stage an increase in the reticulum of the follicles could be demonstrated, and the pulp cords were shrunken so that the sinuses appeared unduly widened and prominent. A striking feature was the alteration of syncytial nuclei from the plump oval endothelial type into elongated forms resembling the nuclei of fibroblasts or of fibrocytes. A marked fibrillary increase could also be demonstrated (fig 12), so that the occurrence of numerous venous spaces bounded by condensed and fibrotic pulp cords suggested the "fibro-adenic" of the Banti spleen in man (fig 13). Capsulo-trabecular thickening and fibrous spread was variable so that the fibrous alteration in

## CIRRHOIC SPLENO-MEGALY

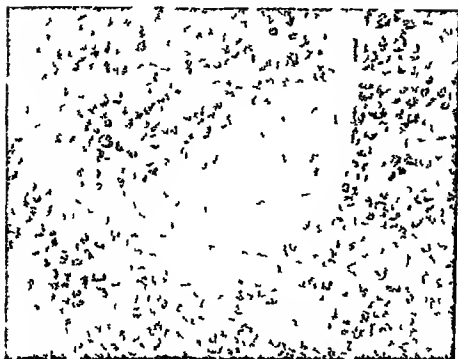


FIG 7—Rat spleen after 45 injections of 0.1 cc of carbon tetrachloride. Extramedullary hyperplasia and formation of nucleated cells. H and E  $\times 300$

FIG 8—Guinea pig spleen after 3 injections of 0.1 cc carbon tetrachloride. Distension of pulp vein with mononuclear and neutrophilic leucocytes; note inflammatory cells in pulp. H and E  $\times 310$

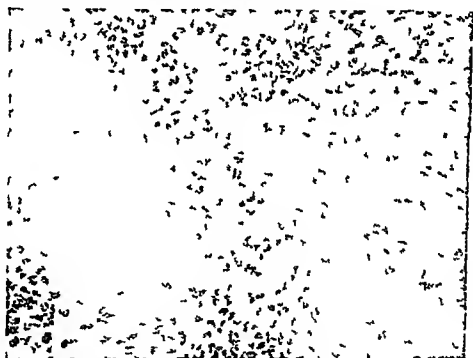


FIG 9—Rat spleen after 2 injections of selenocaine showing early fibrillary increase at marginal zone of follicle. Foot Wilder  $\times 230$



## CIRCUMSCRIBED SPLENOGALIA



FIG 10—Rat spleen after 3 in. of splenogalia. Micrograph of fibrillary structure. Foot Width  $\times 1.0$

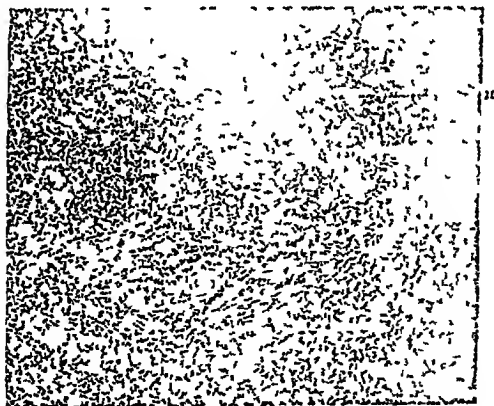


FIG 11—Rat spleen after 2 injections of 1 m. of splenogalia. Micrograph of fibrillary structure. Foot Width  $\times 1.0$





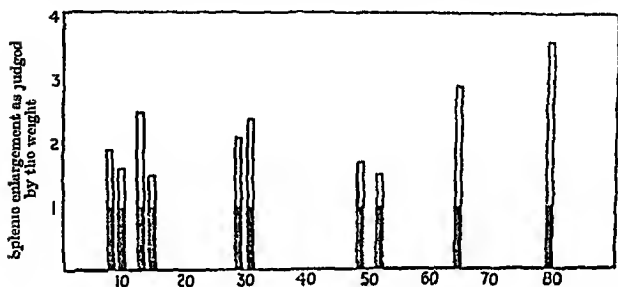




the pulp could not be regarded as an extension from the capsule and trabeculae but was due to some other factor acting intrinsically on the pulp. The changes in the liver were similar to those described by Davidson with retrorsine.

### *Repeated small doses*

After 10 to 16 injections of senecionine in doses gradually increased from 2 to 5 mg twice and thrice weekly the spleen showed well marked enlargement up to three or four times the normal size (text fig 3). This was due to a remarkable proliferation of the



TEXT FIG. 3.—Showing the splenic enlargement in rats after repeated injections of senecionine. The black columns represent the estimated normal weight of the spleen. The abscissa show the total amount of the drug administered (in mg.)

reticulo endothelium of the marginal zone of the follicles (fig 14) as well as of focal areas of the pulp. The lymphoid aggregations in the perivascular and peritrabecular zones were much larger than normal and had spread out more or less diffusely into the pulp. The pulp cords appeared richer in nuclei. In places, focal collections of pale staining syncytial nuclear groups seemed to be the points of activity from which proliferative changes spread to the pulp. Many of these foci were overrun however with lymphocytes and basophilic cells resembling plasma cells, suggesting the possibility of a common origin from the syncytium. Congestion was slight and diffuse and there was no stasis in the sinuses. Erythrophagocytosis was marked. Megakaryocytes appeared unduly numerous.

In the liver the appearance was that of diffuse toxic damage followed by regeneration. There was marked hypertrophy and hyperchromasia of the Kupffer cells and the endothelium of the hepatic venules showed remarkable hyperplastic activity. Lymphoid cells and plasma cells had formed dense clusters around the bile ducts and portal venules and had extended between the

lobules, together with thin strands of collagen in the manner of a portal cirrhosis.

**Summary.** 1. In acute senecionine intoxication in rats there is syncytial necrosis especially of the marginal zone of the malpighian follicles of the spleen

2 With subacute intoxication there is a collapse sclerosis and fibrillary overgrowth in the periarterial zone of the malpighian follicles followed by a gradual fibrillary increase in the pulp These reactions resemble the "fibro-adenic" of the malpighian follicles and pulp cords seen in Banti's disease.

3 With repeated small doses there is a marked splenomegaly due to hyperplasia of the reticulo-endothelium of the pulp At this stage the liver shows marked endothelial proliferation of the hepatic veins, hyperplastic changes in the Kupffer cells and early portal cirrhosis

## DISCUSSION.

### *Toxic splenomegaly.*

Analysis of the various stages of toxic action demonstrates that splenomegaly is pre-cirrhotic, being present in the early stages of liver necrosis. In human pathology similar splenic enlargements have been described associated with acute and subacute liver "atrophy" (necrosis), but they have been looked upon as reactive or congestive in nature Experimental work has shown, however, that degenerative changes in the spleen are common though not well defined, owing to the diffuse structure of the syncytium Just as patterns of necrosis can be distinguished in the liver, so there is a splenic pattern with localisation of necrosis in the marginal zone of the malpighian follicles where the arterial capillary system opens out into the pulp A perivascular lesion is often met with, whilst some follicles are completely destroyed

Regenerative reactions develop in the follicles, and with smaller doses and in the stage of recovery these changes are well marked Hence there arises a type of splenomegaly due to the toxic agent acting directly on the spleen as well as on the liver Sometimes extreme hypertrophy of the malpighian follicles follows a prolonged period of rest (5 weeks) after repeated follicle damage The lymphoid mesh work is converted into a germ-centre type of tissue from which it is assumed the lymphocytes arise. This widespread proliferative reaction with enlargement of the follicles to three or four times the normal diameter and alteration to a more embryonic cell type resembles the condition described in man as "giant lymph follicle hyperplasia" (Brill, Baehr and Rosenthal, 1925) and "lymphoid reticulosis" (Ross, 1933) conditions which are regarded as simple (Ross, 1933) or neoplastic (McNee, 1934) types of reticulo-endotheliosis. On the other hand proliferation may be

more diffuse, involving the secondary lymphoid foci in the pulp. Small foci of reticulo endothelium seem to be activated. This effect was especially marked with repeated small doses of senecionine. In two cases the reaction was so marked after carbon tetrachloride injections that the histological picture suggested a monocytic leukaemia but the other organs showed no similar infiltration. In the later stages of cirrhosis produced, for example, by carbon tetrachloride in rats, the splenic reaction changes in type through the effects of portal stasis.

### *" Fibro adenie "*

The experimental method also throws light on the development of " fibro-adenie " of the malpighian follicles. This lesion is typically perivascular unless toxic damage is extreme. It differs from replacement fibrosis in that fibroblastic growth from the vessel wall is slight and secondary. A collapse sclerosis is followed by a collagenous transformation of the reticulum fibrils as well as by increased overgrowth of the reticulum. Many lymphocytes are destroyed in the necrotic process and in the later stages the follicles gradually become acellular and fibrous. The change is most marked around the penicillar arterioles as well as the large pre follicular arterioles, where the " fibro adenie " can be seen as a denudation of fibrous tissue on one side of the follicle or between neighbouring follicles. The distribution of the lesion and the morphology of the fully developed stages are similar to what Banti (1910) described in man, though he denied the inflammatory nature of the process.

### *The significance of the hæmorrhages*

The association of arterial and periarterial necrosis with focal areas of hæmorrhage into the perimalpighian zone after two or three injections of senecionine raises important questions. Such an acute hæmorrhagic lesion obviously cannot be regarded as due to chronic venous stasis followed by rupture. On the other hand, there is a severe toxic necrosis of the capillary walls at the marginal zones where the arterial capillaries open out into the pulp. The hæmorrhage seems to be due to leakage through the damaged vascular endothelium. In acute carbon tetrachloride poisoning in man, the gastric hæmorrhage and the petechie in other organs can be better correlated with the toxic action of the drug than with venous stasis. A similar mechanism in the production of the siderotic nodule and in splenic mæmia cannot be dismissed. McMichael (1931) has stressed the fact that the fibrotic periarterial lesions in splenic mæmia contain blood and that the fibrosis is the result of previous hæmorrhage followed by organisation. However the present study suggests that this need not necessarily be the

case, as the hæmorrhage may be the result of a toxic vascular lesion in a necrotic patch.

*Pathogenesis of the splenic fibrosis and proliferation.*

Cameron and Karunaratne have advanced a theory of hepatic cirrhosis that appears to have an analogy in the pathogenesis of the splenic lesion. They suggest that products of autolysis of the hepatic cells are drained by the lymph spaces to the portal tracts where they stimulate fibroblastic growth. In the earlier stages, liver destruction is compensated for by regeneration of the liver cells until a stage is reached when such regeneration is inadequate. If we now turn to the spleen during the toxic reaction we find the early development of a periarterial necrosis, provided the dosage has been sufficient to affect the more resistant mesenchymal tissue. As in hepatic cirrhosis, while the lesions induced by smaller doses are compensated by regenerative proliferation, with repeated toxic damage there is a tendency for the persistence of the lesion, with gradual spread of fibroblastic tissue from the periarterial zone. It seems therefore that there is an essential similarity between the splenic reaction and the process of repeated destruction and repair in hepatic cirrhosis.

The effect of a smaller dose in producing proliferative growth of splenic tissue also requires explanation. Reticulo-endothelial stimulants capable of inducing proliferative reactions are well known to be present in extracts of splenic tissue. While there is no agreement as to the mode of action of these extracts (Danilewsky, 1895, Pearce, Krumbhaar and Frazier, 1918, Eddy, 1921, Leake, 1923-24 *a* and *b*, Leake and Leake, 1923-24, Leake and Evans, 1924), all are agreed that after injection they induce increased hæmopoietic activity. Louros (1928), in a study of the reticulo-endothelium-stimulating activity of various substances, has found that splenic extract and splenic lipid are the most potent. It seems probable that repeated focal damage is followed by the liberation of growth-promoting substances which act on the cells of the spleen. Evidence for the subsequent splenic hyperactivity is shown, not only by an increase of splenic tissue, but also by the remarkable erythrophagocytic activity that is usually present. Further, in some cases the resulting destruction of red cells is compensated by an over-production in the bone marrow, well shown by Smyth, Smyth and Carpenter (1936) in carbon tetrachloride intoxication in rats.

CONCLUSIONS.

- 1 With the chronic intoxication induced in animals by manganese chloride and by the alkaloid senecionine, the marginal

zones of the malpighian follicles and the pulp cords show necrosis of the pulp synectium followed by collapse and overgrowth of the fibrillary reticulum and collagenisation. These changes are most marked around the small arterioles, further there is an extension of fibrous tissue from the adventitia of the vessels into the affected areas. The lesions thus produced are more or less similar to the periarterial fibrosis and fibrosis of the pulp which Banti described under the term "fibro adenoma."

2 With the development of these changes there is increased erythrophagocytic activity in the spleen and spleno-megaly which is quite marked even in the early stages and which is independent of hepatic cirrhosis.

3 Carbon tetrachloride induces a marked proliferative reaction, with formation of numerous mononuclear cells in the spleen during the early stages of liver damage. In the later stages, with the development of portal cirrhosis there is a superadded congestive reaction in the spleen.

4 Hyperplastic reactions in the malpighian follicles are found with very small doses of these poisons and bear a resemblance to the condition described as "lymphoid reticulosis" in man.

5 The experimental evidence suggests that during the development of hepatic cirrhosis both portal and biliary there are fibrotic and proliferative reactions in the spleen and a definite spleno-megaly independent of portal stasis. During the toxic process there are changes comparable with those seen in spleno-megaly.

The author desires to thank Professor A. Murray Drinnan for his helpful criticism. This work was supported by a grant from the Earl of Moray Fund of Edinburgh University.

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## THE VACCINATION OF GUINEA-PIGS WITH LIVING BCG, TOGETHER WITH OBSERVATIONS ON TUBERCULOUS SUPERINFECTION IN RABBITS

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It was our original intention to make a series of systematic observations on the superinfection of tuberculous animals. Before describing these and the reasons that led to their abandonment it may be well to define our terms.

The term *superinfection* is used by such workers as Lange (1929) and Selter, Fetzner and Wiklund (1934 a and b) to refer to infection with virulent tubercle bacilli of an animal that has previously been inoculated with avirulent tubercle bacilli. Other workers such as Burke (1935) and Pagel (1936) prefer the word *reinfection* for this process. Neither of these terms seems to us to be strictly applicable to a process that is essentially the infection of a vaccinated animal. Whether the immunising inoculation is made with dead bacilli or with living avirulent bacilli is immaterial, the animal is a vaccinated animal and the test dose of living virulent bacilli provides the first opportunity for the exposure of the animal's tissues to fully infective material.

The term *superinfection* is best limited to infection of an animal which has previously been infected with bacilli of the same type and of the same order of virulence, and in whose tissues these bacilli are still living. It is essentially a process in which something qualitatively similar is added to what is already there. In this sense the expression is correctly employed, for example, by Boquet and Laporte (1937).

The term *reinfection* should be confined to infection occurring in an animal which has previously been infected with bacilli of the same type, but in whose tissues the original bacilli or their descendants have probably died out. It is essentially a process in which a previously but no longer infected animal receives a fresh infection.

In practice it will seldom be possible to differentiate with certainty between superinfection and reinfection. Nevertheless as the reaction of the animal may differ in the two instances, it is well to have separate terms by which we can refer to these conditions.

The aim of our experiments was to reproduce in laboratory animals the superinfection, or possibly reinfection, of the lungs with fully virulent bacilli that we know to occur naturally in human beings.

*Superinfection experiments in rabbits*

A number of rabbits were infected intratracheally with a total of about 4000 virulent bovine bacilli. Seven to nine weeks later half of them were left untreated, half of them were superinfected intratracheally or intravenously with a dose of 500-5000 bacilli of the original strain, while a similar number of untreated animals were inoculated intratracheally or intravenously at the same time in order to control the superinfecting dose. The animals were observed for another six weeks. When a rabbit died, a corresponding animal in each of the other two groups was killed, so that the progress of the lesions in the three groups of animals could be compared. Besides noting the extent and severity of the macroscopic lesions, quantitative lung cultures were put up from every animal, using the technique described by Schwabacher and Wilson (1936-37a).

The results were profoundly disappointing. The extent of the disease in the superinfected and the original animals was sensibly the same, and neither a study of the macroscopic lesions nor of the lung cultures suggested that the primary infection had had any appreciable effect on the progress of the second infection. It was evident that the original infecting dose was too high, and that if any useful observations were to be made in laboratory animals on superinfection, a much less severe disease would have to be established.

Accordingly we undertook a study of the effect of minimal doses of tubercle bacilli on rabbits, guinea-pigs and mice. The results of this investigation, which have already been published (Schwabacher and Wilson, 1936-37a), revealed the fact that in none of these animals was it possible to set up in the lungs a chronic retrogressive disease by the inoculation of virulent mammalian tubercle bacilli—using the term virulent in relation to the species of animal studied. If the numbers were too small no infection occurred, if they were large enough to cause infection, then the animals developed a progressive and ultimately fatal disease. We were therefore forced to the conclusion that the ordinary laboratory animals are quite unsuited for the study of superinfection of the lungs with mammalian tubercle bacilli. It was for this reason that we ultimately directed our attention to following the course of the disease in animals which had been previously vaccinated.

Reference should perhaps be made to the observations of Lange and Lydén (1929) on superinfection of guinea-pigs with mammalian tubercle bacilli. Both the primary and the superinfecting doses consisted of fully virulent bacilli, but the superinfecting dose was given intracutaneously. Under these conditions the superinfected animals proved more resistant than the controls. So far as an attempt to understand the results of pulmonary superinfection or reinfection in human beings is concerned, this method is of more academic than practical interest. Even if it can be shown that the lesions set up by bacilli inoculated into the skin are less severe in a superinfected than in a normal animal, it does not follow that a

similar result would be obtained in the lung. Indeed there is clinical evidence to suggest that the acute inflammatory reaction resulting from the superinfection of the lung in human beings may be followed under certain conditions by dissemination or even generalisation of the disease.

To reproduce a picture strictly analogous to pulmonary tuberculosis in man, in whom a virulent human or bovine bacillus may give rise to any form of the disease from a completely latent infection on the one hand to a fulminating primary tuberculosis on the other, will require the use of animals other than those commonly used in the laboratory. Until such animals are found, and until observations on superinfection and reinfection of the lung with virulent tubercle bacilli can be made, we doubt whether a study of tuberculosis in laboratory animals is likely to throw much further light on the pathogenesis of human pulmonary tuberculosis.

### *Vaccination of guinea-pigs with BCG*

Three experiments were performed

#### *Experiment I*

Twenty normal guinea pigs were inoculated in one thigh with 5 mg. of BCG. Thirty-eight days later they were divided into four groups of five and inoculated intramuscularly into the opposite thigh with virulent bovine bacilli. Viable counts were made on each of the inocula using 1% medium.

*Group 1* received one dose of 0.001 mg. containing about 65,000 viable bacilli.

*Group B* received two doses at weekly intervals of 0.0005 mg. containing a total of about 65,000 viable bacilli.

*Group C* received eight doses at weekly intervals of 0.00025 mg. containing a total of about 230,000 bacilli.

*Group D* received eight doses at weekly intervals of 0.000125 mg. containing a total of about 120,000 viable bacilli.

In each group five unvaccinated control guinea pigs of approximately the same weight were inoculated at the same time with the same dosage of virulent bovine bacilli. Whenever an animal died in one group a corresponding animal was killed in each of the other groups in order to compare the extent and severity of the tuberculous lesions in the organs. In this way all animals had died or been killed within 15 weeks of inoculation with the first test dose. Graded intradermal tests with old tuberculin were made 30 days after inoculation with BCG and on five occasions after infection. The reactions were read 24 hours later. A reaction was regarded as positive unless one or other of the two following criteria was fulfilled: (1) the diameter of the combined area of redness and induration was 20 mm. or over; (2) there was an area of redness and induration measuring 15 mm. in diameter, unaccompanied by central blanching or necrosis.

*Results* Thirteen animals died in the control series and 7 were killed, 7 animals died in the vaccinated series and 13 were killed. *Post mortem* the lesions were situated mainly in the lymphatic

*Superinfection experiments in rabbits.*

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TABLE I  
Exp I Showing highest dilution of tuberculin to which animals reacted

	No tested	Vaccinated						No tested	Control					
		No reacting to							No reacting to					
		1 10	1 50	1 100	1 1000	1 10 000	1 10		1 50	1 100	1 1000	1 10 000		
30 days after B C G vaccination	20	2	4	14	17		20	0						
14 days after infection	20			7	10		20	1	10					
4 weeks after infection	10				10		20							
0	19				10		17							
11	18				4	14	17					20	1	
12	3				3		8					10	0	
							3					2	1	

TABLE II

Exp II Results of inoculation with virulent human tubercle bacilli of guinea pigs vaccinated with two doses of 2 mg of B C G at 27 days' interval (All animals except one, died within the period of observation)

Group	No. of guinea pigs	Infesting dose in mg and no. of doses	Total no. of viable bacilli inoculated	Survival time in days		No showing tuberculous lesions p.m.	Extent and severity of lesions			No. of colonies of tubercle bacilli in lung culture	
				1 M	8 L		++	+	Nil	3 strains	0 M
							++	+			
A Vaccinated Control	5	0.000001 (1)	21	204.4	29.63	4	3	1	0	44 000	960 > 10 <sup>6</sup>
B Vaccinated Control	5	0.000001 (1)	21	183.0	18.97	5	5	0	0	08 > 10	920 > 10 <sup>6</sup>
C Vaccinated Control	5	0.000000.5 (2)	20	175.0*	71.16	4	2	0	1	14 000	100 > 10 <sup>6</sup>
D Vaccinated Control	5	0.000000.25 (4)	20	188.0	31.18	5	2	0	0	4 9 > 10 <sup>6</sup>	4900 > 10 <sup>6</sup>
E Vaccinated Control	5	0.000000.125 (8)	22	230.0	3.91	4	3	0	1	0	310 > 10 <sup>6</sup>
F Vaccinated Control	5	0.000000.0625 (16)	32	182.2	19.06	4	5	1	0	1 7 > 10 <sup>6</sup>	2110 > 10 <sup>6</sup>
G Vaccinated Control	5	0.000000.03125 (32)	72	177.2	6.11	4	0	0	1	0700	670 > 10 <sup>6</sup>
H Vaccinated Control	5	0.000000.015625 (64)	72	171.6	78.14	4	3	0	0	14 > 10 <sup>6</sup>	58 > 10 <sup>6</sup>
I Control	5	0.000000.0078125 (128)	72	171.6	78.14	4	3	0	0	14 > 10 <sup>6</sup>	180 > 10 <sup>6</sup>

1 M survival time of vaccinated group = 181.2 days

8 L survival time of control group = 60.6 days

Observed difference = 120.6 days

1 M - antituberculous

Standard error of this difference

Observed difference divided by its standard error = 1.61

1 M - antituberculous

Observed difference after 2.5 days

glands and to a less extent in the spleen and liver, as a rule the lungs were only slightly affected. Little difference was noticeable between the different groups of vaccinated animals, but the lesions in the control animals were on the average more extensive and severe than those in the vaccinated.

The tuberculin sensitivity of the animals is recorded in table I. Except for the first fortnight after infection, there was little difference between the vaccinated and the control animals in the rate at which tuberculin sensitivity was attained or in the degree which it reached.

From this experiment it was concluded that BCG afforded slight protection, but that the size of the infecting dose used was too great to allow any substantial difference between the vaccinated and control groups to be revealed.

### Experiment II.

Twenty normal guinea-pigs weighing 150-250 g. were inoculated intramuscularly in the left thigh with 2 mg of living BCG. The dose was repeated after 27 days. The total dosage of BCG received by each animal was therefore 4 mg. Thirty-six days after the second vaccination the animals were divided into four groups of five. Along with five control animals for each group they were inoculated with virulent human tubercle bacilli into the right thigh.

*Group A* received one dose of 0 000001 mg

*Group B* received two doses at weekly intervals of 0 0000005 mg

*Group C* received four doses at weekly intervals of 0 00000025 mg

*Group D* received eight doses at weekly intervals of 0 000000125 mg.

A viable count was performed on each inoculum. The aggregate number of viable bacilli given to each group was extremely small, varying from about 20 to 32.

The animals were kept in separate cages. Every fortnight the size of the inguinal glands on the right side was determined by palpation. Graded intradermal tuberculin tests were performed at frequent intervals. All animals were left to die, except one in group B which was killed after 352 days. At necropsy the extent and severity of the lesions present were carefully noted. In addition the lungs were ground up and cultivated in such a way as to provide an approximate estimate of the number of living bacilli present.

*Results* Palpation of the right inguinal region showed that increase in size of the glands occurred earlier in the control than in the vaccinated series. Thus, five weeks after infection—counting from the first dose in groups B, C, and D—the right inguinal gland could be palpated in 11 out of 20 of the control and in only 1 out of 20 of the vaccinated animals, seven weeks after infection the corresponding numbers were 13 and 6, and thirteen weeks after infection 15 and 6. In some of the vaccinated animals the glands did not become palpable for 18-37 weeks. Altogether 12 of the vaccinated and 17 of the control animals showed enlargement of

TABLE I  
Exp I Showing highest dilution of tuberculin to which animals reacted

	Vaccinated					No tested	Control						
	No reacting to						No reacting to						
	1 10	1 50	1 100	1 1000	1 10000		1 10	1 50	1 100	1 1000	1 10000		
30 days after BCG vaccination	20					20							
14 days after infection	20		14			20							1
4 weeks after infection	10	4	3			20						20	0
0 "	10			17		10			10			16	0
11 "	18			10		10						8	0
15 "	3			4	14	17	0					2	1

TABLE II

Exp II Results of inoculation with virulent human tubercle bacilli of guinea pigs vaccinated with two doses of 2 mg of BCG at 27 days interval (All animals, except one, died within the period of observation)

Group	No of guinea pigs	Infecting dose in mg and no of doses	Total no of viable bacilli inoculated	Survival time in days		No showing tuberculous lesions p.m	Extent and severity of lesions				No of colonies of tubercle bacilli in lung cultures		
				A.M	S.F		+++	++	+	Nil	Extra mcs	0 M	
A	Vaccinated	5	0 000001 (1)	284 1	20 63	5	3	1	1	0	44,000	100 × 10 <sup>4</sup>	12 × 10 <sup>4</sup>
B	Control	5	0 000001 (1)	183 0	16 07	5	2	0	0	0	08 × 10	920 × 10 <sup>4</sup>	190 × 10
	Vaccinated	5	0 000000 (2)	275 0*	31 10	4	2	2	0	1	14 000	4900 × 10 <sup>4</sup>	92 × 10
C	Control	5	0 000000 (2)	188 0	34 16	4	6	0	0	0	19 × 10 <sup>4</sup>	310 × 10 <sup>4</sup>	62 × 10
	Vaccinated	5	0 000000 (4)	350 6	35 01	4	3	1	0	1	0	2110 10	31 × 10 <sup>4</sup>
D	Control	5	0 000000 (5)	162 2	10 03	5	5	0	0	0	17 × 10 <sup>4</sup>	070 × 10 <sup>4</sup>	62 × 10
	Vaccinated	5	0 000001 (8)	277 2	26 44	4	0	0	1	1	0700	58 × 10 <sup>4</sup>	0.2 × 10
	Control	5	0 000001 (15)	171 6	36 11	5	3	2	0	0	11 × 10 <sup>4</sup>	180 × 10 <sup>4</sup>	51 × 10

A.M survival time of vaccinated group = 46.6 days.

S.F survival time of control group = 181.2

(Observed difference)

Standard error of this difference

Observed difference divided by the standard error

20.0 days

1.01

S.F = standard error of mean

\* Use subject &amp; died after 32 days

G.M = geometric mean

the glands at some time or other. The remaining data are recorded in tables II and III.

Table II shows that the average survival time in days of the vaccinated animals was significantly greater than that of the control animals. It will be noted that three of the vaccinated animals showed no macroscopic lesions *post mortem*. The spleen of each of these animals was ground up and inoculated into a normal guinea-pig. None developed tuberculosis. Lung cultures of the original animals, however, showed that tubercle bacilli were present in small numbers in two of them; the third animal had apparently resisted infection completely. In the remaining animals the lesions were, on the whole, more severe and extensive in the control than in the vaccinated series. In every control animal lesions were present in the lymphatic glands, spleen, liver and lungs. In the 17 vaccinated animals showing lesions, the lymphatic glands were involved 16 times, the lungs 17 times, the spleen 15 times and the liver 13 times. Again, whereas 18 out of 20 of the control animals had lesions described as "severe" or "massive" in one or more organs, only 8 out of 20 of the vaccinated animals had lesions of this degree of severity.

Reference to the last column of table II shows that the geometric mean number of tubercle bacilli that could be cultivated from the lungs at death was as a rule very much higher in the control than in the vaccinated series.

Whether judged by rate of enlargement of the inguinal glands, average survival time, extent and severity of lesions at necropsy or numbers of living bacilli in the lungs at death, there is no question that the vaccinated animals in this experiment proved more resistant than the controls. Thus increased resistance was, however, relative, and was seldom sufficient to protect the life of the animal or to bring about complete destruction of the tubercle bacilli in the tissues.

Examination of table III brings out the interesting fact that the rate of development of tuberculin sensitivity and the ultimate degree of sensitivity reached were both greater in the control than in the vaccinated animals. By the seventh week after infection, for example, all the control but only one of the vaccinated animals reacted to 1:1000 old tuberculin. Again, 18 of the 20 control animals ultimately became sensitive to 1:100,000 tuberculin, whereas not one of the vaccinated animals did so. It is interesting that the vaccinated animal in group B which was found, *post mortem*, to be apparently free from tuberculosis nevertheless reacted 19 weeks after infection to a 1:10,000 dilution of tuberculin. It is possible that this degree of sensitivity arose from the BCG vaccination alone or that the animal became infected, but succeeded in eliminating the infecting organisms completely.



TABLE III  
 Exp II Showing highest dilution of tuberculin to which animals reacted

	No tested	Vaccinated							No tested	Control						
		No reacting to								No reacting to						
		1 10	1 100	1 1000	1 10 000	1 100 000	1 10	1 100			1 1000	1 10 000	1 100 000			
At time of infection	20	14	6					20	0							
3 weeks after infection	20	9	11					20	10	1						
7       "       "	20		10	1				20			20			18		
13       "       "	20			13	7			20				2				
19       "       "	20				20			14				14				
24       "       "	17			17				8			7	1				
37       "       "	11				11			1					1			
43       "       "	0				0			0								

### Experiment III.

In this experiment the number of doses of B C G was varied, while the infecting dose was kept constant. The animals were a little heavier to commence with than those in exp. II, weighing 270-370 g. Only one group of control animals was included

*Group A* received a single inoculation of 1 mg of B C G.

*Group B* received five inoculations each of 0.2 mg of B C G at weekly intervals

*Group C* received ten inoculations each of 0.1 mg of B C G at weekly intervals

All inoculations were made into the left thigh. One week after the last inoculation of group C with B C G, all animals, together with six controls, were inoculated intramuscularly into the right thigh with 0.000001 mg of virulent human bacilli. A viable count on the inoculum showed that this dose contained about 5 living organisms. After infection the animals were treated like those in exp. II

*Results* The right inguinal glands became enlarged more regularly and more rapidly in the control than in the vaccinated series. Thus five weeks after infection the glands were palpable in 5 out of the 6 controls, but in none of the 18 vaccinated animals, after eight and thirteen weeks all the controls were affected, whereas the numbers in the vaccinated group were 2 and 12 respectively

Table IV shows that the average survival time was significantly greater in the vaccinated than in the control animals

None of the animals escaped infection completely. In one animal of group A the lesions were confined to the lymphatic glands and lung cultures were negative. On the whole the lesions were more severe and extensive in the control than in the vaccinated group. Thus in the control group lesions described as "severe" or "massive" were found in the lymphatic glands, spleen, liver and lungs in every animal. In the 18 animals of the vaccinated group the lymphatic glands were involved 16 times, the lungs 15 times, the spleen 17 times and the liver 14 times. In only 11 of the 18 vaccinated animals were the lesions described as "severe" or "massive"

Except in group B the geometric mean number of tubercle bacilli that could be cultivated from the lungs was considerably less in the vaccinated than in the control animals. So far as group B is concerned, it may be noted that every animal was still alive after all the controls had died

As in exp. II, it may be concluded that the vaccinated animals proved more resistant than the controls

Examination of table V shows that the rate of development of tuberculin sensitivity was greater in the control than in the vaccinated animals. Moreover, 5 out of 6 of the control animals ultimately became sensitive to a 1:100,000 dilution, while none of the vaccinated animals did so.

TABLE IV

Exp III Results of inoculation with virulent human tubercle bacilli of guinea pigs that had been vaccinated with a total dosage of 1 mg of BGG (All animals, except one, died within the period of observation)

Group	No of guinea pigs	Vaccinating dose and no of doses	Approximate no of virulent tubercle bacilli inoculated	Survival time in days		No showing tuberculous lesions post mortem	Extent and severity of lesions			No of colonies of tubercle bacilli in lung cultures	
				A.M.	S.L.		+++	++	+	Extremes	G.M.
A	0	1.0 mg (1)	5	190.5	10.20	6	5	0	1	0	1020 10*
B	0	0.2 " (6)	5	204.2	10.10	5	5	1	0	104 000	8700 x 10*
C	8	0.1 " (10)	5	224.0*	32.53	0	1	3	2	10 000	309 x 10*
Controls	0		5	149.8	13.07	0	6	0	0	230 x 10*	4.2 x 10*
										240 10*	72 x 10*

A.M. survival time of vaccinated group = 220.2 days  
 A.M. survival time of control group = 140.8  
 Observed difference = 70.4  
 Standard error of this difference = 19.8 days  
 Observed difference divided by its standard error = 3.9

G.M. = geometric mean

S.E. = standard error of mean

\* One animal killed after 337 days

TABLE V

Exp III Showing highest dilution of tuberculin to which animals reacted

	No tested	Vaccinated						No tested	Control					
		No reacting to							No reacting to					
		1 10	1 100	1 1000	1 10 000	1 100,000			1 10	1 100	1 1000	1 10 000	1 100 000	
At time of infection	18	6	12	2	1		6	0						
24 days after infection	18	1	14	3			0	0						
5 weeks after infection	18	1	14	3	5		0	0	3					
0 " ,	18		4	0	13		0	0						
15 " "	17			5			0	0			1	5		

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Examination of table V shows that the rate of development of tuberculin sensitivity was greater in the control than in the vaccinated animals. Moreover, 5 out of 6 of the control animals ultimately became sensitive to a 1:100,000 dilution, while none of the vaccinated animals did so.

obtaining as satisfactory protection with a dead vaccine as with living BCG, provided adequate attention is paid to dosage and other factors. There is little exact information to guide us here, but the observations of Petroff and Stewart (1926) and of Pagel (1937) on guinea pigs, of Clawson (1933-34) and Opie and Fround (1937) on rabbits, and of Schwabacher and Wilson (1936-37b) on mice lend some support to this inference.

As regards frequency of vaccinating injections, there was a slight suggestion in exp. III (table IV) that the animals receiving ten doses of 0.1 mg. of BCG were better protected than those receiving one dose of 1 mg. The number of animals, however, was too small for the difference to be significant. The same suggestion was apparent in the mouse experiments already referred to. Thus it was found that in one experiment mice receiving twenty doses of 1 million bacilli were better protected, in relation to the controls, than mice in another experiment receiving four doses of 10 million bacilli. Even if these differences were significant, however, it would be dangerous to lay down any general law regarding frequency of dosage without far fuller inquiry.

It is interesting to note that the average number of colonies of tubercle bacilli growing from the lungs of animals which had died was generally very much less in the vaccinated than in the control animals (tables II and IV). It must be remembered, however, that the average extent and severity of the visceral lesions in the vaccinated animals were less than in the controls. Taken in conjunction with the observations of Jensen, Bindslev and Holm (1935) on vaccinated guinea pigs infected by inhalation, and with those of Schwabacher and Wilson (1936-37b) on mice, there seems to be little doubt that vaccination with BCG either interferes with the development of virulent tubercle bacilli in the body or else enables the tissues to kill them off more rapidly. The experimental observations of Lurie (1936) suggest that both factors are at work.

The effect of vaccination with BCG in retarding the rate at which tuberculin sensitivity was developed after infection with virulent bacilli and in diminishing the ultimate degree of sensitivity reached is evident from tables III and V. These results are of considerable interest. They suggest that the allergic response of a vaccinated animal to infection is very different from the antibody response. Glenny and Sudmersen (1921) and Glenny (1925, 1931), it will be remembered, drew attention to the rapid rise in antitoxin titre that occurs after the injection of toxin into an animal which has already received a preliminary dose. The effect of the first stimulus is greatly to augment and accelerate the reaction after the second. This has since been found to be true of other types of antibodies. The effect on allergy, however, appears to be just the reverse. Instead of rising more rapidly and to a higher level

*Discussion.*

The results obtained in our experiments on the protective effect of vaccination with B C G in guinea-pigs against subsequent inoculation with virulent bacilli are, on the whole, similar to those reported on guinea-pigs by workers such as Krause and Willis (1926), Okell and Parish (1928), Lange (1929), Lange and Lydtin (1929), Birkhaug (1933), Selter, Fetzner and Weiland (1934 *a* and *b*), Corper, Damerow and Cohn (1936), and on rabbits by such workers as Clawson (1933-34) and Lurie (1936), using either B C G or some other attenuated strain of bacillus for vaccination. It seems clear that under suitable conditions the average survival time of vaccinated animals is longer and the average extent and severity of the lesions less than in normal animals. Under exceptionally favourable conditions of time and dosage it is possible to obtain complete or almost complete protection of a certain proportion of the animals, but this degree of success has been recorded by only a few workers.

The *dosage of the vaccinating bacillus* appears to be of some importance, at any rate so far as B C G is concerned. In exp II the average survival time of the animals receiving a total dosage of 4 mg of B C G was 262 days as compared with one of 226 days in the animals of exp III which received a total dosage of only 1 mg. This difference is only 1.7 times its standard error and, though suggestive, cannot be regarded as significant. In a series of experiments on mice, however, using much larger numbers of animals it was found that the best results were not obtained until a dosage of 20 million bacilli (0.02 mg) was reached (Schwabacher and Wilson, 1936-37b). Increasing the dose above this level seemed to confer no additional advantage on the mice. Similarly Okell and Parish obtained their best results on guinea-pigs with a dose of 20 mg. Increasing the dose to 100 mg did not appear to be of any greater value. In this connection it will be remembered that Topley (1933), in a series of vaccination experiments on mice using killed *Bact. typhi-murium*, found that the protective effect of the vaccine, which was given in a dose of 500 million bacteria once a week, increased up to a total dosage of 3000-4000 million, and then diminished as this dosage was exceeded.

The fact that relatively small doses of B C G are less effective than larger doses is difficult to explain except on the ground that B C G. does not multiply to any considerable extent in the mammalian body. In experiments made to determine this point Lurie (1934) found that slight multiplication did occur, particularly in the lymphatic glands, after intravenous inoculation of B.C.G. into rabbits, but that the organisms usually underwent fairly rapid destruction. If this is so, one would expect it to be possible to

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in the vaccinated than in the control animal, tuberculin sensitivity appears to be delayed and impeded in its development by vaccination. Discussion on the interpretation and significance of this phenomenon, as well as on the relation of allergy to immunity, had better be postponed to a subsequent paper in which the effect of sensitisation and desensitisation on the development of experimental tuberculosis is described.

### *Summary and conclusions*

1 The terms superinfection and reinfection are defined

2 An attempt to set up by infection and superinfection a disease in the lungs of rabbits resembling the chronic type of pulmonary tuberculosis met with in man was completely unsuccessful

3. Experiments were carried out on the infection with virulent bovine or human tubercle bacilli of guinea-pigs that had been previously vaccinated with living B C G.

4 With large infecting doses there was little difference between the vaccinated and the control animals

5 With small infecting doses the vaccinated animals differed from the controls in the following respects (a) the regional lymphatic glands increased more slowly in size and often failed to become palpable at all, (b) tuberculin sensitivity took longer to develop and did not reach so high a level, (c) the average survival time was significantly longer, (d) the average extent and severity of the lesions at necropsy were less, and (e) the geometric mean number of colonies of tubercle bacilli cultivated from the lungs *post mortem* was considerably smaller

6 These results show that under suitable conditions vaccination with B C G may confer a certain amount of protection, in occasional animals even complete protection, against subsequent infection with virulent bacilli. The immunity conferred is, however, of a comparatively low order, and it is doubtful whether it is superior to that attainable by suitable injections of dead bacilli. In the present experiments the vaccinated animals were usually unable to withstand completely the inoculation of even minimal numbers—5 to 30—of virulent tubercle bacilli

7 It seems probable that the effect of vaccination with B C G in guinea-pigs is to retard rather than to suppress the development of tubercle bacilli in the body, and thus to favour the occurrence of a more chronic type of the disease

8 Particular interest attaches to the difference in skin sensitivity to tuberculin in control and vaccinated animals.



## THE PREPARATION OF GOLD SOLS FOR THE LANGE TEST

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ALTHOUGH a considerable literature exists concerning the preparation and use of colloidal gold for the Lange test and although the test has received world wide recognition and is extensively used in clinical work, many workers still experience difficulty in consistently preparing a satisfactory sol, and some indeed have consequently been forced to abandon its use altogether. Colloid systems other than gold have also been tried, but these have their own peculiarities and none is as sensitive or as reliable as a well prepared gold sol.

Owing to its undoubted value and world wide use they without consideration that the preparation of the sol was worth investigating as a purely colloid problem. The facilities for colloid investigation provided by the Johnson Chemical Laboratories of the University of Adelaide were accordingly utilised whilst the necessary pathological control tests were carried out by one of us (R. J. C.) at the Institute of Medical Science, Adelaide. The object of the work was to place the preparation of a reliable sol on a scientific basis in order to eliminate as far as possible the chance precautions which ordinarily make the work both tedious and difficult. Between 1800 and 2000 separate gold sols were prepared before the authors were satisfied that they had developed a simple routine preparation, which with practice and moderate care could be made to yield comparable sols with only very occasional failure.

Although the method offers a considerable advance in simplicity of control and in reproducibility of exactly similar sols, nevertheless like all colloid methods it can never be followed without due care. For instance, it is the inviolable practice in colloid laboratories to work with vessels of resistance glass and to keep them completely clean. To depart from this rule is to ask trouble.

The problem divides itself naturally into two parts, the preparation of a stable yet sensitive sol whose particles are of uniform size and uniform surface charge, and second the standard sensitisation (where necessary) of this sol so that it will give a matter what its age, a positive Lange test with particles of size



14 to 22  $\mu$  according to the conditions of preparation. A simple calculation shows that 5 c.c. of a phosphorus reduced sol, whose particles are of diameter 3  $\mu$ , contain the same number of particles (diameter 15  $\mu$ ) as 1 litre of formaldehyde reduced sol. Accordingly if one wishes to prepare a litre of formaldehyde reduced sol with particles 15  $\mu$  in diameter one adds to the hot solution before the addition of the formaldehyde 5 c.c. of a phosphorus reduced sol, thus introducing the required number of nuclei which if all grow, will be equal to the number of gold particles in the finished sol. By varying the amount of phosphorus reduced sol or by using the same amount of stronger preparations, one can vary at will the size of the particles in the final product.

The assumption is made that under the above conditions no fresh nuclei are formed and the ordinary conditions are thus completely reversed. The presence of the foreign colloid material instead of being undesirable now becomes an asset, as it prevents the formation of fresh nuclei. Water that has been once distilled in order to free it from electrolytes and general impurities is thus the best for preparation purposes and for the same reason traces of colloid matter picked up subsequent to distillation will have no great harmful effect.

This view is borne out by practical work which clearly shows that small traces of foreign matter in once distilled water have no deleterious effect upon the crystallisation of the gold or the matter on the stability of the sol or on its sensitivity in the Lange test. Reasonably pure water is of course essential and all preparations must be carried out with considerable care. The possibility of obtaining a clear and stable sol are obviously greatly increased by the simplified technique and elaborate precautions are no longer necessary.

#### *The phosphorus reduced sol used for seeding*

This sol is very easily prepared, and a half a litre of it is sufficient to seed 100 litres of formaldehyde reduced sol, its introduction does not add to the technical difficulties. In several attempts were made to use this sol directly for the Lange test but it proved much too stable. For seeding purposes however it is excellent, and it can be kept from 6 to 8 months or longer if the usual storage precautions are observed.

The phosphorus reduced sol is best prepared (see section III) with a total strength of 100 mg. of gold per litre, when with formaldehyde reduced sols are of the usual strength, namely 0.05 g. per litre. This must be kept in mind in the calculations.

It is recommended that 5 c.c. of the phosphorus reduced sol be the standard amount used in preparing one litre of formaldehyde reduced sol. This gives a useful fairly stable sol whose particles

a negative test with normal fluids. These considerations are discussed in the first two sections of this paper, whilst the third comprises a summary of practical instructions.

## I PREPARING THE SOL AND REGULATING THE SIZE OF THE PARTICLES.

Gold sols for the Lange test are usually prepared from pure gold chloride, using formaldehyde or oxalic acid as the reducing agent. The formaldehyde method, apart from giving a very sensitive sol, has some advantages peculiar to itself (see below), and was accordingly adopted as the standard throughout this work.

Ordinarily, as soon as the formaldehyde is added to the hot solution, a conflict arises between the velocity of formation of gold nuclei and the velocity of crystallisation of the gold. In order to produce a useful sol the formation of nuclei must be encouraged. For many years it has been realised that the merest trace of certain colloid matter can prevent the rapid formation of nuclei, in which case the crystallisation velocity takes control and an unstable and useless product results.

Much previous work has centred round the task of removing these unwanted colloids, and it has been the practice to remove them by a second or even a third distillation of the water used in the preparation. Even so the quality of the water must vary in degree as it can never be wholly colloid-free, and even when useful sols are obtained the latter will show variations among themselves in the number, size and reactivity of the particles (Thiessen, 1929). Apart from this, harmful colloids often enter the water after distillation and cause troubles which are difficult to place.

As there is no *a priori* reason for believing that the colloid material has any other ill effect than that of over-emphasising the crystallisation velocity at the expense of nuclei formation, it appeared to us that the introduction of a certain number of gold nuclei before the addition of formaldehyde would be a satisfactory corrective step which would remove the necessity for rigorous elimination of foreign colloid material; by this means it was hoped to circumvent its harmful effect. Furthermore, by varying the number of nuclei, a series of sols could be prepared whose particles would be of any desired diameter and of uniform size. The very minute particles of a phosphorus-reduced sol at once suggested themselves as suitable nuclei for this purpose. This sol has the advantage of being easily prepared, and the particles are so small (diameter about  $3\text{ m}\mu$ ) that for the purpose they can be considered, as a fact used, as nuclei (Zsigmondy, 1906).

particles of the ordinary formaldehyde-reduced sol as under the ultra-microscope have diameters varying from

## PREPARATION OF GOLD SOLS

14 to 22 m $\mu$  according to the conditions of preparation. A simple calculation shows that 8 c.c. of a phosphorus reduced sol, whose particles are of diameter 3 m $\mu$ , contain the same number of particles (diameter 15 m $\mu$ ) as 1 litre of formaldehyde reduced sol. Accordingly if one wishes to prepare a litre of formaldehyde reduced sol with particles 15 m $\mu$  in diameter, one adds to the hot solution, before the addition of the formaldehyde, 8 c.c. of a phosphorus reduced sol, thus introducing the required number of nuclei, which if all goes well is equal to the number of gold particles in the finished sol. By varying the amount of phosphorus reduced sol, or by using the same amount of stronger preparations, one can vary at will the size of the particles in the final product.

The assumption is made that under these conditions no fresh nuclei are formed and the ordinary conditions are thus completely reversed. The presence of the foreign colloid material, instead of being undesirable, now becomes an asset, as it prevents the formation of fresh nuclei. Water that has been once distilled in order to free it from electrolytes and general impurities is thus the best for preparation purposes, and for the same reason traces of colloid matter picked up subsequent to distillation will have no great harmful effect.

This view is borne out by practical work which clearly shows that small traces of foreign matter in once-distilled water have no deleterious effect upon the crystallisation of the gold on the nuclei, on the stability of the sol, or on its sensitivity in the Lange test. Reasonably pure water is of course essential, and all preparations must be carried out with considerable care. The possibilities of obtaining a clear and stable sol are obviously greatly increased by the simplified technique, and elaborate precautions are necessary.

### *The phosphorus reduced sol used*

This sol is very easily prepared, and as little as 100 mg. is sufficient to seed 100 litres of formaldehyde reduced sol. Its introduction does not add to the technical difficulties. In earlier attempts were made to use this sol directly for the Lange test, it proved much too stable. For seeding purposes, however, it is excellent, and it can be kept from 6 to 8 months or longer if the usual storage precautions are observed.

The phosphorus-reduced sol is best prepared (see section III) with a total strength of 100 mg. of gold per litre, whereas the formaldehyde reduced sols are of the usual strength, namely 50 mg. per litre. This must be kept in mind in the calculations.

It is recommended that 5 c.c. of the phosphorus reduced sol be the standard amount used in preparing one litre of formaldehyde-reduced sol. This gives a useful, fairly stable sol whose particles

have a diameter very close to  $14\text{ m}\mu$ . Smaller amounts of the seeding solution will do, but more should not be added. If more nuclei are required before a clear, bright sol is produced, it is better to start afresh and to take more care in preparing the water, as obviously the colloid matter present is now interfering. Owing to the natural ageing suffered by all colloid systems, the seeding solution shows a slight decrease in efficiency with time. To correct for this, one should add about 5 per cent for each month the sol has been made.

## II REGULATING THE SENSITIVITY OF THE SOL.

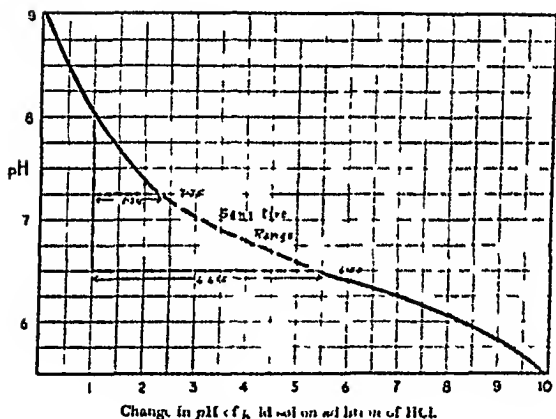
From time to time the important influence of the  $pH$  on the sensitivity of the sol has been stressed, for example, by Mellanby and Anwyl-Davies (1923). The best results are obtained in the Lange test when the sol is practically neutral. Now it so happens that when formaldehyde-reduced sols are prepared in the ordinary way, say by the method of Cruickshank (1920), the finished sol is very close to neutral, due to a fortuitous circumstance which is not generally appreciated. Before the addition of the formaldehyde (Cruickshank's details) the  $pH$  of the mixture of auric chloride and potassium carbonate is in the neighbourhood of 10.1. When the formaldehyde is added, some of it is immediately oxidised to formic acid by the auric chloride, but when the whole of the latter is reduced to the metal, the excess formaldehyde is catalytically oxidised to carbonic acid at the surface of the colloidal gold particles. This latter action, which of course steadily brings down the  $pH$ , proceeds only in alkaline solution, accordingly it gradually slows down as the  $pH$  approaches 7. This is exceedingly convenient, and it gives the formaldehyde method a unique advantage.

However, unless the  $pH$  is actually measured, this behaviour may give rise to a false security, for there is an opposing action, namely the steady escape of  $\text{CO}_2$  from the hot solution, which tends to increase the  $pH$  value. As is seen from the curve in the accompanying figure, the sol is not well buffered above  $pH$  7.2. In odd cases, owing to variations in the governing conditions, the sol may finish with a  $pH$  nearer 8 than 7, when it will be too stable to give the ideal paretic curve. If, however, the  $pH$  is measured and adjusted such a sol responds immediately.

Whilst it is advisable always to prepare the gold sol in exactly the same way, faithfully following the specified conditions given below, it is essential to measure the  $pH$  of each preparation and if necessary adjust it to within the sensitive range. For fresh sols this lies between 6.5 and 7.25, where as it happens the sol is fairly well buffered (table I and figure). A rapid and simple method of  $pH$  adjustment is described in section III.

TABLE I  
Titration of gold sol with HCl

Amount of 0.1 N HCl added to 100 cc sol	pH
0 cc	10.0
1	9.03
2	7.33
3	7.01
4	6.79
5	6.55
6	6.40
7	6.26
8	6.09
9	5.82
10	5.46



### The ageing of the sol

When the sol is kept for any length of time the pH slowly rises, due to the slow escape of  $\text{CO}_2$ . This increase in pH with age makes the sol less sensitive, and unless the pH is adjusted it may fall out of the sensitive range and give rise to trouble. On the other hand certain ageing phenomena common to all colloids gradually reduce the stability of the sol, as is shown by a slight fall in electrophoretic velocity and in surface charge. This interpreted means that the sensitive pH range of any one sol slowly rises and that adjustment, if any need not be so severe.

It must be stressed that there is no absolute value of the rise

sensitive pH range for the Lange test. The most suitable range is chosen arbitrarily from figures such as those shown in table II. Some workers might prefer to work at a slightly higher range than that indicated. There is one guiding rule however. Once a sol gives trouble, its pH should be at once tested to see if it has moved outside the sensitive range. If tests show that it has not done so, then the sol has become contaminated and should be thrown away.

TABLE II.

*Variations in the Lange response with variations in pH*

pH of gold sol	Paretic fluid	Normal fluid
8.36	2, 2, 3, 3, 3, 1, 0, 0, 0, 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
7.70	5, 5, 4, 3, 3, 2, 0, 0, 0, 0	0, 0, 1, 0, 0, 0, 0, 0, 0, 0
7.16	5, 5, 5, 4, 3, 2, 0, 0, 0, 0	0, 0, 1, 0, 0, 0, 0, 0, 0, 0
6.71	5, 5, 5, 4, 3, 2, 1, 0, 0, 0	0, 0, 1, 1, 0, 0, 0, 0, 0, 0
6.40	5, 5, 5, 5, 3, 2, 1, 0, 0, 0	0, 1, 1, 2, 1, 0, 0, 0, 0, 0
6.20	5, 5, 5, 5, 3, 2, 1, 1, 0, 0	1, 1, 2, 2, 1, 0, 0, 0, 0, 0
5.88	5, 5, 5, 5, 5, 3, 2, 1, 0, 0	1, 1, 2, 2, 1, 0, 0, 0, 0, 0
5.42	5, 5, 5, 5, 5, 4, 3, 2, 1, 0	1, 1, 2, 2, 1, 0, 0, 0, 0, 0

Incidentally, the sols described in this work have small particles of uniform size and are stable for several months. Moulds, if they develop, should be neglected, or the sol decanted, on no account should the solutions be filtered.

### III SUMMARY OF PRACTICAL INSTRUCTIONS

*The water.* The best water for the purpose is that which has been distilled once through a copper, tin or Pyrex condenser. An ordinary soft glass condenser can be used but is not recommended. Twice-distilled water will give very stable sols whose sensitive range is a little lower than normal. The water which is now used regularly in these laboratories comes from the continuous electro-osmosis plant of the Johnson laboratories or from the condenser of the boiler room of the Adelaide Hospital. It receives no further treatment before use. There is no need to use freshly distilled water as is so often mentioned. In fact water is improved on standing for some time in a stoppered Pyrex flask.

*The reagents.* With the older (non-seeding) method of preparation it was essential to use the very best of reagents. In our work high grade reagents were used as a matter of course, but latterly we have made an exception in the case of gold chloride, preferring to use the less expensive commercial samples of this salt. No difference whatsoever was detected during either the preparation or the subsequent handling of the sols.

*The apparatus.* As with all colloid work the apparatus should be of resistance glass and should receive a preliminary washing with hot chromic acid before being put into service. After use it should be washed, rinsed with distilled water and put on the racks to dry before storing in a dust-free cupboard. This applies particularly to the test-tubes in which the Lange tests are carried out.

*The stock phosphorus-reduced sol used for seeding.* This sol should be freshly prepared every 6-8 months, or more often if necessary, using the



following procedure. A saturated solution of phosphorus in pure ether is first prepared and the ether solution diluted with a further four volumes of the solvent. (The diluted solution should ultimately be well stoppered and stored away, as it will keep for years.) In 500 c.c. of distilled water at room temperature are added 10 c.c. of a 1 per cent mercuric chloride solution followed by 7.5 c.c. of 2 per cent potassium carbonate solution and the whole well mixed. Then 5 c.c. of the ethereal solution of phosphorus is stirred into the mixture, which slowly begins to turn brown and after 24 hours is either brown or deep red. If it is boiled for a short time after at the end of 15 minutes or 24 hours after the addition of the phosphorus it should turn a deep cherry red. If the brown colour in transmission light persists after boiling it is an indication that the water is not sufficiently pure.

Usually the sol is very easy to prepare. The authors used once distilled water and commercial gold. However it will be noticed that in this case the gold has to form its own nuclei and it would probably prove advisable always to use twice distilled water for this particular preparation.

*The bulk formaldehyde reduced sol.* It is best to make this sol in batches of 500 c.c. and then mix the batches together. This makes for easier control and also smooths out any slight inequalities which may arise. The method of preparation adopted by the authors is as follows. 500 c.c. of once distilled water is placed in a litre Pyrex beaker over a hot burner. When the temperature has reached 50-60°C. 1 c.c. of 2 per cent mercuric chloride is added, followed by 1 c.c. of 2 per cent potassium carbonate solution and the mixture stirred. When the temperature has almost reached the boiling point 1.5 c.c. of the stock phosphorus reduced sol are added slowly with steady stirring. Then with the liquid gently boiling 1 c.c. of 1 per cent formalin (or 5 c.c. of 0.4 per cent formaldehyde) are added, the stirring being continuous. A motor stirrer was used in our work and proved very efficient, but a piece of 3/4 in. Pyrex tubing sealed at one end serves reasonably well. It is essential to keep the mixture thoroughly stirred or shaken during the reduction.

Special attention must be paid to the manner in which the formaldehyde is added, since the conditions of reduction associated with the presence of the formaldehyde determine the uniform nature or otherwise of the sol particles. First add 5 drops of the formaldehyde solution, then wait for one minute or until the colour that is developing reaches a maximum and a second 5 drops and again wait. Then add a third 5 drops and at the end of a further minute the colour should be quite deep. Ten drops can then be added slowly, when it will be noticed that the colour undergoes a rapid and characteristic change to a lighter shade. This is an indication that the preparation has proceeded normally and that the particles are now of approximately even size. The rest of the formaldehyde can be added directly and the preparation at once removed from the flame. Unless this technique is adopted the quality of the sol is sure to vary.

If the sol is cloudy or shows the least trace of turbidity transmission light it should be rejected. It is not advisable to try and correct a poor sol by adding more of the seedling solution, the better plan is to start afresh and use better water.

*Measuring and adjusting the pH.* Before using the sol its pH must be taken. Employing a glass electrode, this can be done by the indirect method in the following way. Prepare a freezing mixture of crushed ice and salt in a 500 c.c. beaker, stir thoroughly until the temperature is about -15°C. Pipette 20 c.c. of the gold sol into a wide test tube (diameter 1 cm.) securely and plunge into the freezing mixture. Leaving it until fairly cold

at least half an hour. Remove and thaw the sol, when its red colour will have disappeared, being replaced by a weak blue. The latter slight colour is due to coagulated particles which have not yet settled. These particles do not seriously interfere with the colorimetric measurement of the pH, using phenol red as indicator. This is an unusual but very satisfactory method.

If the pH of the fresh sol is less than 7.25, the sol can be used directly, but if higher it should be adjusted to within the sensitive range, namely, pH 6.5 to 7.25. The adjustment is carried out in the following way. From the curve in the figure, one reads at sight the number of c.c. of 0.1 N HCl which must be added to one litre of sol to lower its pH over any desired range. For instance, if the pH is initially 8.0, one sees from the curve that one litre of sol will require from 1.3 to 4.4 c.c. of 0.1 N HCl to bring the pH within the sensitive range, 7.25 to 6.5. If the sol is to be used at once, it is best to adjust to the average pH, say 6.8. It must be kept in mind that the pH after adjustment always rises but never falls.

*Keeping the sol.* All sols show a slow increase in pH with age, due to the escape of  $\text{CO}_2$ . Bulk solutions should therefore be well stoppered, and where small amounts only are to be used, that amount should be poured into a separate container.

The pH of the bulk sol should be taken from time to time. Once it has been adjusted it should last 3 or 4 weeks without further attention, any small increase in pH being roughly balanced by the natural falling off in stability. If the sol is kept longer it will probably need readjusting again, keeping in mind that the older the sol the less severe the adjustment.

*General.* Although many of the stringent precautions can be dispensed with, it must be stressed that the preparations cannot be handled carelessly. The Lange test is a very sensitive one and must be treated accordingly. Where the older technique has been mastered and successful preparations are the rule, one hesitates to suggest any alterations. Nevertheless the authors are convinced that the adoption of the seeding method plus the pH control will not only simplify the technique but will give the careful worker an added degree of confidence.

## SUMMARY.

A method is outlined by which gold sols for the Lange test are prepared without the usual elaborate precautions. The harmful effects of the colloid impurities in ordinary distilled water are counteracted by adding the required number of gold nuclei before the reduction is allowed to proceed. In this way the dimensions of the particles are regulated as desired and the sols formed readily and evenly.

The influence of pH in the Lange test is also stressed. Before use, each sol should have its pH adjusted to lie between 6.5 and 7.25, this being the range over which gold sols can be used. Outside this range the tests are often unreliable. It is shown that the pH may be rapidly estimated by first freezing out the colloid material, and the adjustment (if necessary) rapidly made by use of the titration curve set out in the text.







dose is found to be between 0.048 and 0.093 g. per kg. body weight, with a mean value of 0.064 g., a figure well in agreement with that given by Cushny (1920-24). With intraportal administration, as by injection into a mesenteric vein, the minimal lethal dose varies from 0.08 to 0.28 g. per kg. body weight, with a mean value of 0.15 g. (table I). Such evidence suggests that the liver plays some part in dealing with the alkaloid and this conclusion is borne out by the smaller dose tolerated when the liver is damaged by a poison such as chloroform. Thus in four experiments rabbits were given 1 c.c. of chloroform per kg. body weight subcutaneously. Twenty-four hours later the minimal lethal dose of atropine with portal administration was 0.066–0.083 g. per kg. body weight. Perfusion of the isolated liver with saline containing atropine shows that the liver absorbs much of the alkaloid (Roger, 1922).

TABLE II

*Atropine content of liver following intraportal administration*

Rabbit no	Weight of liver (g.)	Total atropine introduced intraportally (mg.)	Total atropine in whole liver (mg.)	Atropine per g. of liver (mg.)	Proportion of total introduced atropine in liver (per cent.)
1	82	200	30	0.37	15
2	88	210	40	0.45	19
3	66	200	20	0.30	10
4	50	200	50	1.00	25
5	58	200	30	0.52	15
6	110	200	40	0.37	20

That the liver anchors considerable amounts of atropine introduced by the portal route is shown in table II. Fifteen to 25 per cent. of the total amount can be recovered after 2–5 minutes. Only a small portion of this is present in the blood of the liver.

We have estimated the blood content of the rabbit's liver by Schutz's method (1926). The isolated organ is perfused with normal saline at 37° C. until the perfusate is free from blood. If  $X$  = blood volume of liver,  $A$  = volume of perfusate,  $S_x$  = specific gravity of blood,  $S$  = specific gravity of saline,  $S_a$  = specific gravity of the perfusate, then

$$A S_a = X S_x + (A - X) S$$

$$\text{and } X = \frac{A (S_a - S)}{(S_x - S)}$$

Our results show that about 30 per cent. of the liver weight is due to its blood content. Now in a typical experiment after the intraportal injection of 200 mg. of atropine sulphate, the blood as a whole contained 0.05 mg. of atropine per c.c. The liver weighed 50 g. and contained about 15 c.c. of blood, so that of the 50 mg.

of atropine obtained by assay of whole liver tissue only  $15 \times 0.05$  mg ( $= 0.75$  mg) could be attributed to the blood.

It seems certain therefore that considerable amounts of atropine recovered from the liver during the first few minutes after portal introduction are present either attached to the walls of the blood vessels or in the liver cells. It can be readily shown that a great deal of the atropine is loosely combined, for it can be washed out of the vascular channels by simple perfusion. Thus in a fully grown rabbit dying three minutes after the injection of 0.175 g of atropine sulphate into a mesenteric vein, the isolated liver after saline perfusion contained 25 mg, the perfusate 55.5 mg of atropine. The total blood volume of the liver was 33 c.c., the atropine content of the liver blood 2.5 mg, so that most of the atropine in the perfusate must have come from the vessel walls. In several other experiments practically all of the atropine was washed out of the liver in this way. It seems unlikely that much could have been extracted from the liver cells, for there was an intact vascular system. The alkaloid appears, at any rate for the first few minutes, to be loosely attached to the vessel walls of the liver. Thence it is removed, for the atropine content of the organ falls to zero after some hours. Presumably it may be transferred to the liver cells and there dealt with, or removed by way of the tissue spaces (Disse's spaces) to the lymphatics, or further excretion by the blood stream may follow.

The possibility of destruction by the liver cells may be considered first. Since the work of Cloetta (1908) and Clark (1912) it has been generally accepted that emulsions of liver possess the power of rapidly destroying atropine. I have spent some time in repeating this work with variable results.

*Technique.* Following Clark (1912) I grind up 20 g of fresh or perfused liver with sand and 10 c.c. of normal saline to a fine paste. One c.c. samples of this paste are further ground up with 0.5 c.c. of a 0.02 per cent aqueous solution of atropine sulphate, i.e. 0.1 mg atropine sulphate, the mixture well shaken with a few drops of toluol and incubated at  $37^{\circ}\text{C}$  for 148 hours. Emulsion heated for 30 minutes at  $60^{\circ}\text{C}$  before the addition of atropine serves as a control. In general almost the whole of the added atropine can be recovered after 48 hours from this heated emulsion. The alkaloid is assayed by making up each sample to 8 c.c. with saline, adding 5 per cent  $\text{H}_2\text{SO}_4$  to give a 1 per cent  $\text{H}_2\text{SO}_4$  solution and then heating the mixture at  $75^{\circ}\text{C}$  for 30 minutes. After centrifugation the residue is re-extracted with 2 c.c. of 1 per cent  $\text{H}_2\text{SO}_4$  for a quarter of an hour at  $75^{\circ}\text{C}$  and again centrifuged. The total supernatant fluid is treated with 10 per cent NaOH until faintly acid, mixed with five times its volume of absolute alcohol and filtered. The filtrate is evaporated to 1 c.c., filtered, and the residue washed with a few c.c. of 5 per cent HCl. It is made alkaline with NaOH and extracted twice with chloroform. The chloroform extract is separated and evaporated to dryness. The residue is finally taken up in 5 c.c. of distilled water made faintly acid with HCl. Dilutions of this are tested by Vitah's method. I found the most satisfactory way of doing this was to evaporate 1 c.c. of the solution to dryness on a crucible of 18 mm diameter.

allowing it to cool, adding 4 drops of fuming  $\text{HNO}_3$ , evaporating, cooling and adding 3 drops of a saturated solution of caustic potash in absolute alcohol. The end point is taken as the slight violet colouration given by 0.001 mg of atropine sulphate. With tissue emulsions, however, the distinction given between 0.001 and 0.002 mg may be very difficult. Small crucibles are essential for accuracy in dealing with minute amounts of atropine. The caustic potash solution must be absolutely fresh. I have found the method to be highly successful with both small and large (100 g) amounts of liver, nearly 100 per cent of the added atropine being recovered in control experiments.

Unlike Cloetta (1908) and Clark (1912) I have not obtained much convincing evidence that rabbit liver *in vitro* destroys atropine. Even introducing atropine into the organ during life by portal administration before carrying out the *in-vitro* test proved disappointing. In the early stages of the work, when large crucibles were used for Vitali's test, there appeared to be considerable destruction within a few hours, but with the improved technique this could not be confirmed. In two instances, 40 per cent. reduction was quite certain in 24 and 48 hours respectively, but in many more cases there was no destruction. Foetal rabbit liver and regenerated adult liver gave equally unconvincing results. Similarly with rat's liver and human liver no destruction could be demonstrated. Only in the case of the frog were my results in agreement with those of Clark (1912) and von Oettingen (1918), for most of the atropine disappeared in 24 hours. I feel far from convinced therefore that actual destruction by liver cells is an effective method of disposal of the alkaloid.

Moreover, there is no evidence of excretion of atropine in the bile. I am thus forced back upon the possibilities of removal by the lymphatics or the blood vessels, but on these I have no definite information. Several workers (Ozawa, 1929, Pulewka, 1932, Oelkers, Raetz and Rinteln, 1932) have shown that much of the introduced atropine can be recovered from the urine in a number of hours, others (Fleischmann, 1910; Storm van Leeuwen and Zeydner, 1921, Van der Heyde, 1920-21, La Barre, 1925-26; Oelkers, Raetz and Rinteln, 1932) have stressed the importance of destruction or adsorption in the blood. It is difficult to escape the conclusion that renal excretion rather than destruction by the liver or any other organ plays the most important part in dealing with atropine. It does seem certain, however, that if the liver has first call, as with portal introduction, much more atropine is temporarily stored in its vascular labyrinth. Presumably it is given up slowly to the blood stream and excreted by the kidneys. In the absence of preferential treatment the liver shows no excessive storage and the alkaloid is fairly uniformly distributed throughout the tissues. The few analyses I have made show very little difference in the amount of atropine per g of tissue in, for instance, the liver,



kidney and heart, although the brain usually gives very much lower values

### Conclusions

Rabbits tolerate much larger doses of atropine and store greater amounts in their livers with intraportal than with ear-vein administration. Atropine is not excreted in the bile and little is actually destroyed by ground up liver tissue. A great deal of the stored atropine can be easily washed out from the vascular system of the liver. It is suggested that disposal takes place by temporary union with the vessel walls, no absorption into the blood stream and slow excretion by the kidneys. There appears to be little evidence in favour of a specific detoxifying action of the liver against atropine.

I am very much indebted to Professor G. R. Cameron for assistance with the experimental work, and to Dr C. H. Gray for supervision of the chemical details. The expenses were defrayed by a grant from the Ceylon Government.

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## TYPE SPECIFIC BACTERIOPHAGES FOR *CORYNEBACTERIUM DIPHTHERIAE*

E V KEOGH, R T SIMMONS and G ANDERSON

*From the Commonwealth Serum Laboratories, Melbourne*

SEVERAL investigators have isolated, usually with difficulty, bacteriophages acting on *C. diphtheria*. Stone and Hobby (1934) review the relevant literature. This paper is concerned with the correlation of phage susceptibility with biological type and serological group. Using two locally isolated phages, the great majority of local diphtheria strains have been classified in groups which closely correspond with those obtained by other methods (colonial characters, serological reactions, starch fermentation, etc.). During the course of this investigation some hundreds of Australian *gravis* and intermediate strains have been classified serologically, with results in general agreement with those of recent English investigators (Mair 1936; Robinson and Peasey, 1936).

### METHODS

#### *Isolation of phages*

Sewage effluents and faeces from cases of diphtheria were tested for the presence of phage against a number of cultures, singly and in mixtures. Orthodox methods were used and will not be described in detail. In work of this nature one searches more or less in the dark, being uncertain whether the material examined contains a phage or whether the cultures used are susceptible. Two phages were isolated, but it is not certain whether either or both were derived from the excreta or from one or more of the cultures. Later it was found that one of these phages could be obtained regularly from some lysogenic cultures, we have not succeeded in re-isolating the other either from cultures or faeces. These two phages will be designated L (large plaque) and S (small plaque) throughout this paper.

#### *Propagation of phages*

L phage has been grown on an intermediate strain 'Laster'. S phage on a *gravis* strain 'Wray'. It is difficult to obtain high titre filtrates with regularity, especially with 'S' phage. The method finally adopted has been to inoculate 50 c.c. of nutrient broth, to which 10 per cent. of normal horse serum may be added, with a loopful of organisms and simultaneously with 1 c.c. of an active filtrate. After incubation for 13 days the culture is Sartz filtered and the titre of the filtrate determined by enumeration of plaques on agar. If the titre is unsatisfactory the filtrate is re-inoculated



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with the susceptible strain, incubated for 18 hours and re-filtered. This process is continued until a satisfactory titre ( $10^{-8}$ ) is obtained.

The L phage filtrates maintain their titre fairly satisfactorily, falling from  $10^{-8}$  to  $10^{-7}$  in a month at  $0^{\circ}\text{C}$ . The S phage filtrates are less stable as the titre drops rapidly, the filtrate losing 90 per cent of its activity in a week at  $0^{\circ}\text{C}$ .

#### *Production of plaques*

Plates are poured from 1.25 per cent agar prepared by the method of Wright (1933), to which 10 per cent of normal horse serum is added. After a preliminary drying at  $37^{\circ}\text{C}$  for 30 minutes they are spread with an 18-hour broth culture, dried in the incubator with the lids raised, and incubated for another hour with the lids lowered. This preliminary incubation is adopted because it was found that confluent growth on the plates may not result from a broth culture inoculum but does occur if the spreader is run over the plate after 1-2 hours' incubation. Following this incubation the required dilutions of phage are spread using standard technique and the plates dried for a further 15 minutes.

#### *Cultures.*

The majority of the cultures used had been isolated elsewhere and had been typed on isolation. The entire series was retyped, using as criteria (a) colonial appearance on nutrient agar (Wright) to which 0.04 per cent of potassium tellurite and 20 per cent of defibrinated sheep's blood lysed by freezing and thawing are added at  $50^{\circ}\text{C}$  just prior to pouring the plates, (b) starch fermentation, and (c) type of growth in phenol red broth. There were only occasional discrepancies, which will be mentioned in the text, between our results and the findings of those responsible for isolation. Virulence tests had been carried out on these cultures but were repeated, by both intradermal and subcutaneous methods, on all cultures which were atypical in any respect, including the phage and serum reactions.

#### *Agglutination tests*

During the course of some uncompleted studies by one of us (G. A.) on the saline sensitivity of *gravis*, intermediate and *mitis* cultures, which show characteristic differences in this respect, it was noted that all strains examined formed stable emulsions in 1.5 per cent sodium chloride solution. This simplifies the performance of agglutination tests, none of the manoeuvres resorted to by various workers to obtain stable emulsions being necessary. Emulsions are prepared from serum agar slopes in 1.5 per cent saline, by scraping off the growth with a strong nichrome loop and rubbing up well in the saline. If the growth is washed directly off the slope with saline the emulsions obtained, though satisfactory, are less stable. Only six cultures failed to give stable emulsions by this method. The dilutions of serum are also made in 1.5 per cent saline.

Samples of type sera for *gravis* types were kindly supplied by Dr D. T. Robinson, together with cultures of his type strains (Robinson and Peeney). We had also available samples of sera for Mair's *gravis* types A, B, and C and intermediate F. It may be noted in passing that Robinson and Peeney's types I, II, and III correspond to Mair's types A, B, and C.



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*Phage-resistant strains*

Organisms resistant to either or both phages were obtained by the usual methods. Such resistants originating from susceptible *gravis* type II strains show a change in colonial form, the colonies being *mitis*-like in type. We have not succeeded, however, in producing resistant forms of stable colonial type. A *mitis*-like colony, if replated, throws colonies resembling the parent type and typical *gravis* colonies in varying proportions. The instability is still present in the descendant colonies. These *mitis* like colonies resemble the smooth colonial variants of *gravis* strains obtained by Christison (1933) by the ageing of bouillon cultures. We have obtained similar variants by the same method. They are stable in colonial type, differing in this respect from those produced more rapidly by phage action.

None of these resistants showed change in serological type, in power of fermenting starch, in type of growth in phenol red broth or in virulence. But as these resistants were far from stable, the possibility that such variants might be derived by more extensive experiments or with other phages, cannot be excluded.

## DISCUSSION

The above findings show that susceptibility to phage can be used as an auxiliary means of classification of diphtheria strains. In certain cases it may be the only method of characterising a particular strain. The lysogenicity of the Victorian type I strains distinguishes them from otherwise similar cultures which are sensitive to L phage. The Swan Hill type II strains isolated at intervals over a period of nearly a year were characterised by their resistance to both phages. The use of such methods in identification is illustrated by the geographical survey of cholera strains by Bruce White (1937), who used phage sensitivity and lysogenicity to distinguish Chinese and Japanese from Indian strains, and these from the El Tor and other vibrios.

It is improbable that the phages described are the only existing ones. Those previously mentioned in the literature are insufficiently characterised to determine whether they are identical with ours. With these two phages we have successfully classified the local strains of *C. diphtheriae*, but have failed with representative strains (serological types III, IV and V) which have not been encountered locally.

We have no data to indicate whether phage may be implicated in the natural origin of new strains but the possibility exists that phage may play a role in such transformations as may appear spontaneously *in vitro*, or be induced by various methods, e.g. change in colonial type and loss of power of fermenting starch, which

characteristic of the serological group, but is due to their lysogenicity. Tests on the remaining English type strains showed that type II was susceptible to both phages, type III weakly susceptible to L phage only, type IV insensitive to both phages and type V very weakly sensitive to L but insensitive to S. It is evident that with the phages available it is not possible to distinguish the five serological *gravis* types. As we have not found representatives of serological types III, IV and V among the Australian strains it is not surprising that we have also failed to find phages specifically active against them. Search for such phages might be rewarded in countries where these types are common.

(b) *Intermediate strains* Of the 131 intermediate strains examined none was susceptible to S phage; all but two were susceptible to L phage. The titre of L phage was always one hundred-fold higher when tested on intermediate than on *gravis* strains. The two insusceptible strains, which were typical intermediates in all respects including virulence, were shown to be lysogenic in respect of L phage. These strains agglutinate to titre with F (intermediate) serum and are virulent, with 16 exceptions (*vide infra*).

Included in this collection of intermediate strains are 25 cultures previously classified as "atypical *gravis*" by Gregory (1937a), on the grounds that they resemble *gravis* colonially on McLeod's medium and in type of growth in phenol red broth, but do not ferment starch. They correspond to similar strains isolated in Scotland by Wright *et al.* (1935), who designate them *gravis* type IV in their classification. These strains agglutinate to titre with "F" (intermediate) serum (Mair). They resemble typical intermediates in sensitivity to L phage. The colonial appearance of these strains can be readily distinguished from the typical *gravis* strains on the medium we employed, which contains unheated sheep's blood. In view of the serological findings we regard these as intermediate strains.

The 16 avirulent strains in this group were isolated from healthy children by Gregory (1937b). These cultures were all susceptible to L phage but did not agglutinate with F (intermediate) serum, except slightly in low dilutions. They form a homogenous serological group. A serum prepared against one of them agglutinates all the others to titre, but has no action on the virulent intermediates except in very low dilution.

(c) *Mitis strains* None of the 180 *mitis* strains examined is susceptible to either phage. Two cultures, however, were shown to be lysogenic and from them was recovered a phage to which only one culture of those tested is susceptible, the *gravis* strain previously mentioned originating from the Lister Institute.

## AN INTRACUTANEOUS RABBIT TEST FOR THE ASSAY OF ANTIPNEUMOCOCCUS SERUM

JOHN IPSEN, Jr

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ANTIPNEUMOCOCCUS serum is now tested *in vitro* and *in vivo* in many ways which need not be described here. An excellent summary of methods employed up to date was given by Folton and Stahl (1937). In recent years efforts have been made to make the *in vitro* tests as accurate as possible (agglutination, precipitation, N determination in precipitated antibody). Nevertheless, although these methods are quicker, more accurate and less costly than tests on animals, there has been universal reluctance to abandon titration of the protective effect on infection of an experimental animal. It is this effect which is utilised therapeutically, and it has not yet been determined whether or not the *in vivo* and *in vitro* values of antipneumococcus sera are parallel. The tendency to depart from *in vivo* tests is due to the high degree of error and the expense attending them. The practical problem in titrating antipneumococcus serum hitherto has been to procure large numbers of uniform mice but even with a much inbred stock one cannot do with fewer than 10 mice per dose if the limits of error are to be within the range 50-200 per cent. The British Pharmacopœia Commission (1936) has attributed the following grade of accuracy to the mouse protection test: "Limits of error. If 100 mice receive the Standard Preparation and 100 mice receive the preparation being tested the limits of error ( $P = 0.09$ ) are 57 and 176 per cent."

The present paper describes a method of testing the biological effect of antipneumococcus serum in which the drawbacks of the mouse method are avoided, the effect of various serum doses being observed in the *same* animal organism.

*The rabbit intracutaneous test*

(a) *Principle of the method*. A small dose of a highly virulent culture of pneumococcus (types I and II) injected intracutaneously in a rabbit will, in the course of 24 hours, produce intense inflammation appearing in the form of an elevated erythema which spreads widely, both ventrally and laterally. If even large doses of culture are mixed with sufficient type specific antipneumococcus serum, the lesion will fail to appear. This observation forms the basis of the method now described.

Two series of mixtures of a constant dose of live culture and varying doses of (1) a standard serum and (2) an unknown serum are injected into the same rabbit. If it can be assumed that the skin area in which an incipient reaction is observed in the series with the unknown serum contains the same number of units as that which gives a corresponding reaction in

have been reported by various investigators (Christison, 1933; Muir, 1936; Robinson and Peeney, 1936).

### SUMMARY.

1. 180 Australian strains of *C. diphtheriae* have been examined in respect of sensitivity to two strains of phage, S and L.

2. Of these local diphtheria strains, *gravis* type I strains are susceptible to S phage and are lysogenic in respect of L phage, *gravis* type II strains are susceptible to both phages (with 5 exceptions), intermediate strains are susceptible only to L phage (with 2 exceptions which are lysogenic in respect of L phage), *mitis* strains are not susceptible to either phage.

3. A simple method of preparing stable emulsions of *C. diphtheriae* for agglutination is described.

4. The significance of these findings is briefly discussed.

We are very grateful to Mr T. S. Gregory for providing us with a large collection of typed Victorian strains which formed a basis for this work. Large numbers of cultures were also supplied by Professor Harvey Sutton, Dr N. E. Goldsworthy, Dr Helen Kelsoy, and Miss Marjory Candy, to whom we offer our grateful acknowledgments.

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Series A			Series B	
No	Serum (A.U.)	Culture (c.c.)	Serum (A.U.)	Culture (c.c.)
1	0.20	0.005	0.020	0.0005
2	0.16	0.005	0.016	0.0005
3	0.125	0.005	0.0125	0.0005
4	0.10	0.005	0.010	0.0005
5	0.08	0.005	0.008	0.0005
6	0.063	0.005	0.0063	0.0005
7	0.05	0.005	0.005	0.0005
8	0.04	0.005	0.004	0.0005
9	0.032	0.005	0.0032	0.0005

It is difficult to decide which test dose of culture is preferable, perhaps 0.0005 c.c. gives greater accuracy but 0.005 c.c. is more convenient.

(d) *Inoculation of rabbits* Immediately after preparation the mixtures of serum and culture are injected intracutaneously into a depilated rabbit, the standard serum on one side, the unknown serum symmetrically on the other. When making the calculations it must be remembered that only one fourth of the quantity transferred to the glass has been injected.

#### *Reading the intracutaneous reactions*

Antipneumococcus serum has two effects on the injected culture, which in the present work have served as a basis for appraising the potency of the serum. In the first place it prevents the appearance of the conspicuous pathological change in the skin, in the second it inhibits the local growth of pneumococci in the area in which it is injected.

*The anatomical reaction* During the first 46 hours after injection the skin areas present a homogeneous, somewhat elevated erythema which does not permit of any opinion on the subsequent result. After eighteen hours, in areas with the highest serum dose, in typical cases the skin is normal or there is redness without infiltration. The characteristic feature of a positive reaction is infiltration exceeding 10 mm in diameter. In the first, second or third area the infiltration is most often distinctly delimited. Areas with lower serum doses present the typical diffuse reactions and sometimes the largest reactions are completely confluent. There is never any necrosis, but the large reactions are more or less cyanotic in colour.

The best time for reading the result is between 18 and 24 hours after injection. The local reactions are then strongest and most typically developed, and the bacteremia is not yet so pronounced that the general infection confuses the reading. But even at 18 hours, the animals may be in such poor condition, the skin so cold and pale, with reduced turgor, that the reactions are difficult to distinguish.

the standard series, the potency can be recorded in international units. Apart from the skin reaction, the number of live bacteria in each skin area can be used as a test of the potency of the serum

(b) *Technique of the test Culture.* The original Neufeld strain (type I) is used in the form of a serum broth culture about 18 hours old, cultivated direct from the heart of a bacteraemic mouse. Tested on mice such cultures have always proved lethal in a dilution of  $10^{-8}$ . Weaker cultures are useless. Serum bouillon is always employed for dilution.

*Sera.* The international standard serum for type I (series no PA 10 and PA 11) is used in the control series. Against this standard two sera were tested, the potency of which was known from mouse tests. From the National Institute of Health, Washington, Dr Harrison kindly furnished me with samples of the fluid American standard serum P 11. From the National Institute for Medical Research, Hampstead, I received a few ampoules of desiccated horse serum, type 1, selected with the object of replacing the international standard serum type 1, the stock of which was almost exhausted. The contents of one ampoule, representing 5 c c of serum, were dissolved in 25 c c of 66 per cent glycerine-saline, the same dilution as the international standard dilution.

(c) *Preparation of the mixtures.* The titration was carried out with two different test doses of culture, 0.005 and 0.0005 c c, and a range of doses of serum selected so that the skin reaction might be in the middle of the series. As one cannot have more than nine or ten skin areas in one series and as the variation from animal to animal in the quantity of serum required to neutralise a given dose of the same culture is considerable (table I, B), the dose intervals cannot be less than 25 per cent. The following method of preparing mixtures has usually enabled us to attain our object.

From the international standard serum (1 c c = 200 A U) two preliminary dilutions are prepared (A) by mixing 0.2 c c of serum with 9.8 c c of saline (= 1.50) and (B) by mixing 0.2 c c of dilution (A) with 1.8 c c of saline (= 1.500). Dilution A thus contains 4 A U and dilution B 0.4 A U per c c.

From these two dilutions two further series of serum dilutions are made as follows.

Dilution no	Serum dilution A or B (c c)	Saline (c c)
1	0.20	0.0
2	0.16	0.04
3	0.125	0.075
4	0.10	0.10
5	0.08	0.12
6	0.063	0.137
7	0.05	0.15
8	0.04	0.16
9	0.032	0.168

To each tube of the series prepared from dilution A (series A) is added 0.2 c c of culture diluted 1:10 and to each tube of series B 0.2 c c. of culture diluted 1:100. For inoculation 0.1 c c of each mixture is used so that the actual doses employed in the intracutaneous test are as shown in the table (p 573).

The sera compared with the standard were so diluted that the two preliminary dilutions, as judged by the in-vitro and mouse tests, contained the same number of units per c c. as dilutions A and B of the standard serum.

*Accuracy of the rabbit intradermal test*

Table I contains the results of statistical calculations of the variation in the serum dose which permits the occurrence of an unipient reaction with a given dose of culture

TABLE I  
*Accuracy of the rabbit intradermal test*

	Test dose of culture (c c)	Method of reading.	Number of observations.	Degrees of freedom	Standard deviation of logarithm of serum dose	Limits of error (per cent.) (P = 0.99).
A Various cultures rabbits	0.005	Anat	21	20	0.158	39-255
	0.005	Bact	21	20	0.225	28-370
	0.0005	Anat	15	14	0.113	52-195
	0.0005	Bact	15	14	0.154	40-250
B Same culture rabbits	0.005	Anat	10	10	0.157	39-254
	0.005	Bact	19	10	0.199	31-326
	0.0005	Anat	16	9	0.005	57-170
	0.0005	Bact	30	17	0.173	36-270
					Standard deviation of logarithm of A U titre.	
C Evaluation of sera against standard serum in same rabbit	0.005	Anat	21	19	0.109	53-190
	0.005	Bact	21	19	0.105	54-180
	0.0005	Anat	15	13	0.053	73-137
	0.0005	Bact	15	13	0.110	50-109

The first part of the table (A) deals with the variation in the highest serum quantity to permit the occurrence of a reaction as tested against the same dose of various cultures. The second part (B) comprises results of tests made on the same day, when the same mixture of culture and serum was injected into two or three rabbits. The standard deviation and the limits of error are calculated on the difference between the results on the various rabbits. An examination of the distribution of the serum doses makes it extremely probable that only the logarithmic values comply with the normal law of distribution, for which reason the standard deviation is shown in logarithms.

The first part of the table shows that the variation in the quantity of serum which neutralises the test dose from day to day is much greater than when titrating with toxins. This is natural, however, where an agent is concerned which multiplies after injection, and when in addition to the serum the animal organism itself participates in the neutralisation of the agent.

The deviations are so marked that pipetting errors become unimportant, so there is reason for ascribing the great variations mainly to differences in the cultures and in the resistance of the rabbits.

Sometimes there may be doubt as to the area in which the reaction begins, and a subjective element may enter into the adjudication. It might therefore be thought expedient to select the fully developed, typically diffuse reaction as the end point, but the transition from a weaker to a stronger reaction is not more abrupt than that from a negative to a positive reaction, and on the whole the incipient reaction is the best defined titration limit.

*The bacteriological reaction.* For this a technique has been employed by means of which the pneumococci are cultivated from the skin areas, so that one obtains an idea of the relative number of bacteria in each.

Eighteen hours after injection, immediately after the anatomical reaction is read, the rabbit is killed with chloroform. With a sterile razor blade an angular incision is made round the site of injection, the apex is gripped with forceps, and with the aid of another sterile blade the flap of skin is dissected by means of small incisions parallel with the surface through the subcutaneous tissue. The culture is made from the under side of this flap of skin. It is a conspicuous fact that in the positive areas the subcutis is very cedematous and adherent to the underlying tissue, whereas negative areas present a smooth white subcutis and are easily detached.

Cultivation is performed by rubbing a coarse inoculation needle with a small loop ten times or so on the inner side of the flap of skin and transferring the material so obtained to a 10 per cent blood-agar plate  $4\frac{1}{2}$  ins. in diameter, spreading it not too thickly. One quarter of the plate is inoculated from each skin area.

On the following day the typical picture is that only a few colonies, or none at all, have grown from the areas with a high dose of serum, whereas from those with lower doses it is possible to cultivate numerous colonies. The difference between these bacteriologically positive and negative fields is strikingly abrupt. A count of 40 titrations has shown that if from one skin area it has been possible to cultivate "innumerable" colonies ( $>200$ ), the average number cultivated from the preceding area with a 25 per cent. higher dose of serum is 15 colonies, in more than half of the cases the area was sterile. By cultivating four times from the same areas with over 200 colonies the results obtained were constant. From areas with a countable number of colonies this quadruplicate cultivation gave numbers which varied usually from 0 to 50, but were always below 100. There is no correlation between the countable numbers of bacteria and the serum dose.

The end point of the titration in bacteriological reactions will therefore naturally be the highest dose at which a vigorous growth of bacteria occurs, and consequently the comparison between the two sera is quite simple.



TABLE II

*Intracutaneous titration of American standard serum P 11 and new proposed international standard against present international standard*

Serum tested	Test dose of culture (c.c.)	Method of reading	Number of titrations.	Results (A U)	Geometrical mean	Logarithm of mean.	Standard deviation of logarithm.
P 11	0 005	Anat	11	312 250 187, 312, 250, 250, 200 250, 320, 250, 320	262 A U	2 418	0 084
	0 005	Bact	11	200, 200, 200, 200, 250 310, 400, 252, 320 300 320	261 A U	2 417	0 120
	0 0005	Anat	5	250, 250 200, 250, 250	240 A U	2 38	0 048
	0 0005	Bact	5	200 250 200, 200 320	229 A U	2 30	0 089
Proposed standard	0 005	Anat	10	1000 1000 1000, 030, 030 1250, 650 500, 625 025	755 A U	2 88	0 135
	0 005	Bact	10	1000 780 780, 780, 030 1000 625 500 780 040	740 A U	2 87	0 073
	0 0005	Anat	10	780 780 625, 780 025 1000 800, 785 780 800	775 A U	2 89	0 057
	0 0005	Bact	10	035 680, 625 625 500, 1000 625, 625 1000 025	676 A U	2 83	0 126

serum by means of the mouse test was made by Felton and Stahl, with the result that the ratio between the two sera is 300/1150. The international serum was used in the undiluted state. For purposes of definition its titration was put at 1000 A U per c.c. On this basis (80 mice per dose), P 11 should contain  $\frac{300 \times 1000}{1150} = 271$  A U per c.c. This conforms very well with what was found intracutaneously.

*Proposed standard serum.* This is at present being tested by the mouse method in various laboratories in Great Britain. According to a personal communication the preliminary titrations are stated to be 810, about 1200 and about 1600 A U. As will be seen, the results obtained by the rabbit method are lower, 740 A U, but the great diffusion of the results in the mouse test means that as yet we cannot call the difference important.

### Discussion

On the basis of these observations it may be of importance to examine whether it is possible to define the biological reaction which is observed in the rabbit method, and thereby form an opinion as to whether the test results of this method will be of any practical service in determining the value of therapeutic anti-pneumococcus sera.

After eighteen hours two different reactions are observed in the rabbit, (a) inhibition of bacterial growth with rising serum doses, and (b) oedema and infiltration which increase with falling serum doses

Often, but by no means always, the end points of these two reactions coincide. They may differ by several areas, with 25 per cent. difference in the serum dose from one area to another.

In 51 titrations the readings were distributed as follows

	Anatomical end point given with larger dose of serum than bacteriological	End points of both reactions in same skin area	Bacteriological end point given with larger dose of serum than anatomical
Skin areas separating the two end points	3 2 1	0	1 2 3 4 5
Number of titrations	1 3 3	20	11 4 2 0 1

If the difference between the end points of the two methods of reading resulted from an observational error pure and simple, the separation would be at most one or two areas, but seeing that it has been found to be as much as five areas, the necessary assumption is that some biological phenomenon is responsible. Each skin area is injected with the same number of bacteria but the quantity of serum varies. The difference in the reactions is first of all governed by the quantity of serum, but the serum works in conjunction with the natural defence forces of the organism, possibly activated by the serum (Goodner and Miller, 1935).

The number of live bacteria in the field (the bacteriological reaction) will be governed by the growth of bacteria, which is counteracted by bacteriolysis and phagocytosis. How great an effect the serum has on the latter factors will to some extent depend on the responsiveness of the host. The variation of this factor from animal to animal may be judged from the second part of table I.

There is no reason to doubt that the anatomical reaction is due to the pneumococci, but that it is unnecessary to presume that the reaction is due to *live* pneumococci is shown by the fact that in one series it is possible to find up to four anatomically positive areas from which one cannot cultivate more bacteria than from the negative areas on the same animal. The possibility is manifest that the skin reaction may be produced by killed bacteria or bacterial products (toxins?).

Quite schematically it may be said, when titrating with *live* cultures intracutaneously on rabbits, that with the bacteriological reaction one reads the protective effect of the serum, whilst the anatomical end point indicates the sum of the protective and

anti-irritative effects Which of the two properties one should adopt as the criterion of sera for therapeutic use is a question which cannot be settled at this early stage of the investigations

There are grounds for continuing to test with live cultures administered intracutaneously, as it enables us to observe on the same animal two effects of antipneumococcus serum which will possibly lead to a better understanding of the clinical effect Should it turn out that equivalent values are measured by the rabbit and mouse methods, the rabbit method will be quicker, cheaper and more accurate in the routine testing of antipneumococcus sera

### Summary

1 The condition produced in the skin of rabbits by the injection of live pneumococci is inhibited by the addition of type specific antipneumococcus serum in sufficient quantities to the dose of bacteria injected

2 Antipneumococcus serum inhibits the growth of the bacteria when injected with them in sufficient doses

3 A method is described which permits comparison of the effect of varying doses of two antipneumococcus sera on a constant dose of live culture in the skin of the same rabbit

4 The potency in international units obtained by this method approaches that found by the mouse protection test

5 The accuracy of comparing two sera intracutaneously in one rabbit is equal to that of the mouse protection test when 200 mice are injected

6 The nature of the effect of antipneumococcus serum observed by the rabbit intracutaneous method is discussed The two reactions described may be caused by qualitatively different properties of the serum

I wish to thank Dr Harrison and Dr Hartley for kindly providing me with samples of the sera and Dr Kauffmann for allowing me to make use of cultures produced in the daily routine of his department

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# THE HEREDITARY FACTOR IN INDUCED SKIN TUMOURS IN MICE ESTABLISHMENT OF A STRAIN SPECIALLY SENSITIVE TO CARCINOGENIC AGENTS APPLIED TO THE SKIN

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It is a common experience that when a carcinogenic agent is applied to the skin of a number of mice from a mixed stock, there is much individual variation in the date of appearance of both benign and malignant tumours. Eight years ago it was decided to attempt to discover whether these characteristics are heritable, and later, when certain lines of mice had been established, to compare the reaction of the skin with that of mice belonging to some well known inbred stocks. The possibility of the presence of a hereditary factor in the development of induced tumours in mice seemed all the more probable in view of the importance of such a factor in the development of spontaneous mammary and lung tumours.

Kroyberg (1934) described experiments in which, by continuous brother-sister mating of the offspring of one female, he built up a strain of mice (white label) in which benign tumours induced by tar occurred in the population as a whole at a considerably later date than did similar tumours in a strain (red label) already in use in the laboratory and originally derived from Dr Hjalond's stock. A very large number of mice was used and he concluded that the difference was a genetic one. In 1935 he published the results of tarring 1000 members of the white label population and concluded that the reaction as regards the formation of tar cancer was uniform during a period of 5 years but that in the males it was distinctly delayed as compared with the females. In addition he was able to segregate two lines, one with a very high incidence of spontaneous breast cancer and one developing no breast cancer. The latter showed an earlier appearance and higher incidence of tar cancer than the general population, while in the former the appearance was later and the incidence lower.

The tar wart was chosen as a means of demonstrating the hereditary factor because its appearance can be assigned to a definite date, it can be seen readily with the naked eye and although usually benign at first, with the tar in use in this laboratory malignancy almost always supervenes if the animal is allowed to

live for a sufficient length of time. In addition, the conditions of application of the carcinogenic agent can be kept uniform and constant.

The technical difficulties were far greater than were anticipated, as it was found that the long-continued application of tar caused a marked decrease in fertility and diminished the milk supply of the mother. Foster-mothering was considered but was not adopted owing to shortage of accommodation, in view of Bittner's (1937) recent discovery that foster-mothering of litters susceptible to breast cancer by non-susceptible mice can modify the development of breast and lung cancer in the fostered litters, it would seem to have been fortunate that this course was not pursued. Finally it was decided to follow a suggestion of Dr Mottram that the mice should be allowed to breed before tarring was commenced and that the litters should be reserved for further breeding purposes. Thus it would be possible to select the offspring of parents themselves developing early and late warts for the establishment of pure lines.

#### Experiment I. Isolation of the DK strain.

Pregnant female mice were obtained from a dealer and finally 98 were available for the experiment, each with a litter placed in reserve. Tarring of the mothers was then begun. The hair was clipped away with curved scissors from a small area of skin in the region between the shoulder blades. Once weekly this area was painted by means of a camel-hair brush with an ether extract of horizontal retort tar prepared in the following manner. The tar was treated with lime and then extracted with ether in a Soxhlet apparatus. The ether was evaporated and the residue used for application to the skin. The diet throughout the experiments was liberal and mixed.

A wart appeared in each of two mice after 7 and 8 applications respectively. The male offspring of one of these was mated with the female offspring of the other and as far as possible brother-sister mating was continued until the 9th generation was reached (coat colour black, with occasional grey or silver mice). Where no brother was available, either the father or a first cousin was used. The results of tarring of the 9th, 10th and 11th generations were then compared with those of stock mice (table I). All the available mice in each generation were tarred and no selection was made.

Twelve of the original 98 mice did not develop their warts until 27-34 applications of tar had been made, but unfortunately by this time all of the litters were dead, so that no line could be developed from mice with a late tar response.

#### Experiment II. Isolation of the IF and JCFI strains.

In the first experiment no account was taken of the tar response of the male parent, thus it was decided to set up a number of breeding boxes each containing four females and one male. When

a litter from each female had been placed in reserve, tarring was commenced, 105 mice (83 female and 22 male) being available for the experiment. The first warts appeared at 9 weeks, but the only offspring available were those from a mouse developing a wart at 11 weeks, whose mate unfortunately had died during the first few weeks of tarring. Brother-sister mating was instituted among these offspring until the 7th generation was reached, this strain being called IF (coat colour black or grey). The results of tarring of the 7th-11th generations were compared with those of stock mice (table I).

In this experiment, where there was a high mortality among the original mice, all the survivors had developed warts at 26 applications or less but by this time very few of the offspring were surviving. Two females whose female parent developed a wart at 20 weeks and whose male parent had died at 24 weeks without a wart were mated with a male whose female parent developed a wart at 20 weeks and whose male parent had died at 16 weeks without a wart. This strain was called JCFI (coat colour white). It was felt that offspring from parents with a later tar response would have been more satisfactory had they been available. Brother-sister mating was continued until the 7th generation was reached, thereafter the results of tarring of the 7th to the 11th generation were compared with those of stock mice (table I).

### Experiment III

The difficulties of preliminary breeding followed by tarring had proved to be so great that it was decided to make another attempt to isolate a strain with an early tar response by tarring a number of stock mice and breeding from those which developed warts early. Of 50 mice (34 female and 16 male) a female developing a wart at 8 weeks was mated with a male developing one at 11 weeks. The litter thus obtained was very puny and did not survive long enough for breeding to be continued.

#### *Appearance of warts in stock and inbred mice*

In table I are given the times of appearance of warts in stock mice for comparison with those of strains IF, DK and JCFI. The mortality of the stock mice was appreciably higher than that of the inbred mice, and as this mortality was spread over the whole experiment its effect would be to exaggerate the proportion of stock mice developing warts early, because some of the mice which could be expected to develop late warts would already have died. Of the mice of strain IF (selected from a parent with an early wart) 40.2 per cent developed their warts at or before 12 weeks of tarring in contrast with 13.8 per cent of the stock mice. Also a rather

Week	Stock mice			Strain IF (7th-11th generation)			Strain DK (9th-11th generation)			Strain JCFI (7th-11th generation)		
	No of animals developing warts each week	No in groups	Percentage in groups	No of animals developing warts each week	No in groups	Percentage in groups	No of animals developing warts each week	No in groups	Percentage in groups	No of animals developing warts each week	No in groups	Percentage in groups
7	1			0			0			0		
8	2			5			0			0		
9	5			13			1			2		
10	4	22	13.8	21	90	40.2	0	8	4.7	3	13	10.1
11	4			21			1			3		
12	5			30			6			5		
13	6			23			6			8		
14	10	34	21.4	20	72	32.1	8	42	24.7	15	40	31.0
15	10			20			12			7		
16	8			9			12			10		
17	13	45	28.3	12	36	16.1	18	64	37.6	11	43	33.3
18	7			13			17			10		
19	16			7			15			7		
20	9			4			14			15		
21	8			0			10			7		
22	8			2			9			3		
23	6			5			7			1		
24	13			2			5			6		
25	2			2			1			3		
26	10	58	36.5	3	26	11.6	1	56	32.9	2	33	25.6
27	2			1			2			2		
28	4			2			1			2		
29	2			0			0			2		
30	2			0			0			0		
31	1			5*			12*			7*		
32	1											
Total animals developing warts	159			224†			170†			129†		
Total no of mice at beginning of experiment	253			268			203			141		
Percentage of mice which developed warts	62			84			84			91		

\* These mice had not developed warts at the end of 20 weeks of tarring  
† Includes the mice which had not developed warts at the end of 20 weeks of tarring



greater number developed their warts between 13 and 16 weeks of tarring (32.1 compared with 21.4 per cent). After 16 weeks, therefore, 72.3 per cent of the IF mice had developed warts, whereas only 35.2 per cent of the stock mice had done so. It is to be noticed that there is no difference in the range of date of wart development in the two series of mice.

When the DK strain (also selected from parents with early warts) is considered, no corresponding early wart development is found, in fact the warts developed rather later, only 29.4 per cent appearing at 16 weeks or earlier compared with 35.2 per cent in the stock mice. Statistically this difference is not significant. The JCFI strain (selected from parents with fairly late warts) shows somewhat earlier warts than both stock and DK mice.

Berenblum (1931) considers that an estimate of the date at which 50 per cent of survivors show warts gives a fairer index of the rate of wart appearance, as such a method takes into account the mortality during the experiment. Fifty per cent of surviving IF mice had developed warts at 13½ weeks, of DK mice at 18½ weeks and of JCFI mice at 17½ weeks, figures which confirm those already given.

These results show that it is possible by selective breeding to produce mice specially sensitive to the action of tar and that this characteristic is to some extent a genetic one. The fact that only one of the strains showed the characteristic sought for may have been due to chance or to the fact that it was not possible to know the tar response of both parents at the outset. Reference has already been made to the technical difficulties of breeding mice with a late tar response.

#### *Comparison of development of malignancy in three strains of mice bred in the laboratory*

Having established that the mice of the IF strain developed their warts earlier than either stock mice or those of strains DK or JCFI, an analysis of the results of tarring with regard to the development of malignancy was made.

(a) *Interval between wart appearance and development of clinical malignancy* The difficulties of estimating clinically the exact date of the malignant change in warts has often been discussed, notably by Cramer (1936). The criterion which was used here was the first sign of invasion of deeper tissues as estimated by palpation. It is realised that this method allows a considerable error, but it is an error which should apply equally to all the mice irrespective of strain. In table II it is seen that, although the range of the interval between wart appearance and the development of malignancy is not greatly different, the average interval in the strain which

develops early warts is longer by about 4 weeks and 2 weeks respectively than the interval in the other two strains. As the warts appear on an average 4 weeks and 2 weeks earlier in the IF strain, it is seen that the average time from the beginning of the experiment to the date of appearance of clinical malignancy is approximately the same in all the strains, i.e. 23-24 weeks.

TABLE II.

*Interval between wart appearance and development of clinical malignancy*

Strain	No of mice	Shortest interval (weeks)	Longest interval (weeks)	Average interval (weeks)	Average date of appearance of wart (weeks)	Average date of appearance of clinical malignancy (weeks)
IF	77	0	19	10.2	13.2	23.4
DK	82	0	16	6.5	17.4	23.9
JCFI	44	2	16	8.7	15.9	24.3

All the surviving mice were killed at the end of 29 weeks of tarring, a histological examination of the warts of these mice and also of those which died before 29 weeks was made. Areas were chosen which showed the most advanced changes and where necessary several blocks were cut. The usual criteria of malignant change were used, namely hyperchromatism and irregularity of size and shape of nuclei, irregular and abundant mitoses, dedifferentiation of cells, invasion of fat and muscle, etc. It was found convenient to adopt a classification of simple warts, malignant change without invasion (semi-malignant warts, Mottam, 1934) and malignant change with invasion. It is not denied that there is a certain personal factor in diagnosis and classification but to wait for invasion of muscle before admitting the malignancy of these tar warts (Berenblum, 1929) undoubtedly excludes many which have already undergone malignant change.

(b) *Incidence of malignancy (estimated histologically) in relation to the time of existence of the wart*. In this section are included all the mice with warts of which a histological examination was made at autopsy irrespective of the date of death. No mouse was allowed to live longer than 29 weeks. They have been arbitrarily divided into four classes according to the number of weeks before death during which a wart was present (table III). In the IF strain no wart present for 4 weeks or less was malignant, but 2 out of 6 DK warts and 2 out of 9 JCFI warts were already malignant. Similarly only 38 per cent of IF warts which had existed for 5-9 weeks were histologically malignant compared with 75 and 72 per cent of the mice of the other two strains. Of warts which had existed for 10-14 weeks, 55 per cent of the IF mice were malignant in contrast with 86 and 80 per cent respectively of DK and JCFI mice. This lag in the development of malignancy serves to confirm the estimate already made clinically (table II) that the

early appearance of the IF warts is balanced by a longer interval before they become malignant

TABLE III

*Incidence of malignancy (estimated histologically) in relation to the time of existence of the wart*

Strain	Total no of mice	Histology	No in which a wart was present 0-4 weeks.	Wart present, 5-9 weeks.		Wart present 10-14 weeks.		Wart present 15-21 weeks	
				No	Per cent	No.	Per cent.	No.	Per cent.
IF	175	Simple	10	31	03	20	43	19	26
		Mal I*	0	2	38	7	55	0	74
		Mal II*	0	11		25		48	
DK	137	Simple	4	14	25	9	14	2	17
		Mal I	2	3	75	3	86	2	83
		Mal II	0	38		52		8	
JCFI	111	Simple	7	9	28	8	20	9	31
		Mal I	1	0	72	4	80	2	69
		Mal II	1	17		29		18	

\* Mal. I = malignant without invasion      Mal. II = malignant with invasion

(c) *Incidence of malignancy (estimated histologically) after 29 weeks of tarring* The survival rate to the end of the experiment of mice which had developed warts was less than half in strain IF and considerably better in the other two strains (table IV). But in mice which did survive, the incidence of malignancy at the end of 29 weeks is rather less (though not significantly so) in the IF mice than in the others.

TABLE IV

*Incidence of malignancy (estimated histologically) after 29 weeks of tarring*

Strain	No. of mice which developed warts	Mice examined histologically at 29 weeks.		Simple warts		Malignancy without invasion		Malignancy with invasion		Total malignancy	
		No.	Per cent	No	Per cent.	No	Per cent.	No	Per cent	No	Per cent.
IF	210	100	45.7	32	32.0	11	11.0	57	57.0	68	68.0
DK	168	115	72.8	24	20.9	10	8.7	81	70.4	91	79.1
JCFI	122	106	86.0	30	28.3	13	12.3	63	59.4	70	71.7

#### Experiment IV

As the early appearance of warts in the IF strain did not appear to be accompanied by any corresponding early development of malignancy, it was decided to compare it with some other inbred

strains and if possible with strains with a considerably later wart appearance.

I am greatly indebted to Dr Kreyberg for sending me a litter of his "white label" albino mice. Through the kindness of Professor J. B. S. Haldane I also obtained litters of Little's line of Bagg albinos (the females of which have a high incidence of mammary cancer) and of Little's line of black agoutis (CBA) in which mammary cancer is unknown or very rare. After a stock of mice of these strains had been built up by brother-sister mating, tar was applied weekly to the skin between the shoulder blades as described on page 582, and all the surviving animals were killed at the end of 29 weeks of tarring. The degree of inbreeding of white label mice is not known, but the females develop a considerable incidence of spontaneous breast cancer, the Bagg albino mice were in the 45th-48th generation of inbreeding and the black agouti in the 30th-32nd generation.

Lynch (1925) applied tar to the skin of a small number of mice of two strains with varying incidence of mammary cancer and concluded that there was no significant difference in the development of tar tumours. In 1933-34, she published a more extended series of observations and concluded that there were very marked differences in susceptibility to tar-induced skin tumours among various strains of mice. In general the tar tumours were delayed in those strains which developed spontaneous mammary cancer. Reinhard and Candee (1932) found that the response to tarring of two strains of mice, one with a high and the other with a low spontaneous mammary cancer incidence, was similar, except that the latter had a latent period of 12-14 weeks in the development of warts. A similar percentage of warts in each strain became malignant. They noticed also that in the early tar cancer strain (i.e. in the high mammary cancer strain) the females developed warts earlier than the males. Dobrovolskaia-Zavadskaia and Olch (1934), using small numbers of mice of many different lines, found that there was much variation in the response to tar, individual as well as in the strain as a whole, but came to no conclusion as to whether coat colour was an important factor. The same (senior) author with Garrido (1936) extended these observations and found no strain entirely refractory nor was pigmentation of the coat any protection against tar.

It may be stated that the IF mice are very much more sensitive to the development of tar warts than any of these other inbred strains (graph, p. 590). In table V it is seen that 40.2 per cent of IF mice had developed warts by the end of 12 weeks of tarring, whereas no white label (Kreyberg) nor Bagg albino mice and only one black agouti mouse had developed a wart. By the end of the sixteenth week 72.3 per cent of IF mice had warts compared with 8.4, 2.6 and 10.5 per cent. of the other strains. When the date at which 50 per cent of the survivors have warts is compared, there is a difference of from  $6\frac{1}{2}$  to  $11\frac{1}{2}$  weeks, the dates being: IF  $13\frac{1}{2}$  weeks, white label 22, Bagg albino 25 and black agouti 20 weeks.

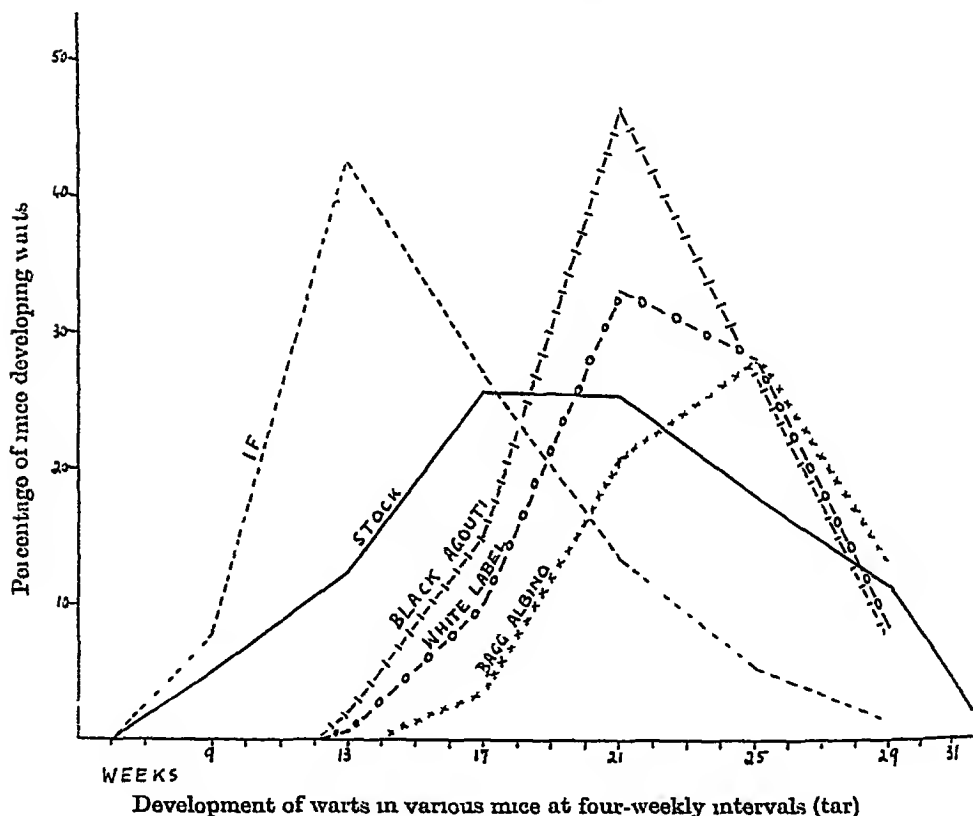
From these data it is seen that the Bagg albino (breast-cancer-susceptible) mice are less sensitive to tar than any of the other strains used. That this lack of sensitivity is not an antagonistic action due to the presence of breast cancer is shown by the fact that none of the mice (of which 65 were female) developed a breast

Week	II			Wistar-Kelley (Karyberg)			Bagg albino (breast-cancer-susceptible)			Black agouti (B1A) (breast-cancer-resistant)		
	No. of animals developing warts each week	No. in groups	Percentage in groups	No. of animals developing warts each week	No. in groups	Percentage in groups	No. of animals developing warts each week	No. in groups	Percentage in groups	No. of animals developing warts each week	No. in groups	Percentage in groups
8	5	90	49.2	0	0	0.0	0	0	0.0	0	1	1.0
9	13			0			0			0		
10	21			0			0			0		
11	21			0			0			0		
12	30	72	32.1	0	12	8.4	0	3	2.0	1	0	0.5
13	23			1			0			2		
14	20			2			1			2		
15	20			0			0			0		
16	0	36	16.1	3	36	2.2	0	14	12.2	0	30	37.9
17	12			8			1			6		
18	13			6			2			0		
19	7			10			4			0		
20	4	26	11.4	14	90	60.4	21	95	8.2	31	40	51.0
21	4			10			0			12		
22	6			10			7			0		
23	5			4			0			0		
24	1	24†		3	13†		2	11†		1	05†	
25	0			20*			0			0		
26	0			0			0			0		
27	0			0			0			0		
28	0	263		143†	159		36*	90		104		
29	0			0			0			0		
30	0	84		0	5		0			0		
31	0			0			0			0		
Total animals developing warts												
Total mice at beginning of experiment												
Percentage of survivors at end of experiment												

\* These mice had not developed warts after 29 weeks of tarring.

† It includes the mice which had not developed warts after 29 weeks of tarring.

tumour before the end of the experiment. The average age of the females at death was 44.6 weeks and all were virgins. It has been found previously (Bonser, 1936) that breast cancers in this strain usually occur in breeding females of 12 months or older and that the incidence is much diminished in virgins. White label (Kreyberg) mice are also liable to develop breast tumours (Kreyberg, 1935). In this experiment 62 females developed warts, 29 were virgins and 33 had borne one or more litters and the average age at death was 50.6 weeks. Sixteen mice developed breast cancer



during the experiment (6 virgins and 10 breeders) at an average age of 43.4 weeks, and the average date of wart appearance in these mice was 20.6 weeks compared with 21.6 weeks in females which did not develop breast cancer. Kreyberg (1935) found that in this strain the individuals with breast cancer showed a considerably later appearance and lower incidence of tar cancer. No information is as yet available as to the incidence of breast cancer in non-experimental mice of this strain but the figures just quoted do not suggest an antagonistic action between the development of breast cancer and the development of tar warts.

*Comparison of the development of malignancy in IF, white label (Kreyberg), Bagg albino and black agouti mice*

(a) *Interval between wart appearance and development of clinical malignancy* Information on this point is given in table VI. Warts take considerably longer to become malignant in IF mice than in the other strains. By contrast, warts in black agouti

TABLE VI

*Interval between wart appearance and development of clinical malignancy*

Strain	No of mice with warts	Shortest interval (weeks)	Longest interval (weeks)	Average interval (weeks)	Average date of appearance of wart (weeks)	Average date of appearance of clinical malignancy (weeks)
IF	77	0	10	10.2	13.2	23.4
White label	43	2	13	9.5	19.1	25.9
Bagg albino	11	4	12	6.9	20.1	27.0
Black agouti	55	0	10	5.2	19.7	21.9

mice become malignant very quickly (average interval 5.2 weeks). Consequently the length of time taken from the beginning of the experiment for malignancy to develop is approximately the same in IF and black agouti mice, but is slightly longer in white label (Kreyberg) and Bagg albino mice.

(b) *Incidence of malignancy (estimated histologically) in relation to the time of existence of the wart (table VII)* No wart present in

TABLE VII.

*Incidence of malignancy (estimated histologically) in relation to the time of existence of the wart*

Strain	Total no. of mice	Histology	Wart present 0-4 weeks		Wart present 5-9 weeks		Wart present 10-14 weeks		Wart present 15-21 weeks	
			No	Per cent	No	Per cent	No	Per cent	No	Per cent
IF	175	Simple	10	100	21	62	26	15	10	26
		Mal I*	0	0	2	38	7	57	0	71
		Mal II*	0		11		25		48	
White label (Kreyberg)	106	Simple	14	70	20	31	4	19	0	
		Mal I	1	30	6	69	3	81	0	
		Mal II	5		38		14		1	
Bagg albino	74	Simple	16	73	23	52	4	50		
		Mal I	3	27	7	18	0	50		
		Mal II	3		14		1			
Black agouti	88	Simple	7	50	5	12	6	21	1	
		Mal I	0	50	6	88	2	79	0	
		Mal II	7		31		21		3	

\* Mal. I = malignant without invasion. Mal. II = malignant with invasion.

an IF mouse for four weeks or less had become malignant, whereas varying percentages of warts of each of the other strains were already malignant, the highest being in the black agouti mice. Of warts present for 5-9 weeks, only 38 per cent in IF mice had become malignant compared with 48-88 per cent of those in other strains, the highest percentage again being shown by the black agouti mice. As the numbers of mice bearing warts for longer than this period become very small in the late strains, statistical comparisons cannot be made, but it is to be noted that of 73 IF warts which had been present for 15-21 weeks, 19, or 26 per cent, were still simple

(c) *Incidence of malignancy (estimated histologically) after 29 weeks of tarring (table VIII)* Even though at 29 weeks IF warts had existed for considerably longer than warts of the other strains, no significantly greater number of them had become malignant, except when compared with the Bagg albino mice, whose warts had only existed on an average for 6.1 weeks. This fact is considered to be a more likely cause of the failure to become malignant than the antagonistic action of the breast cancer susceptibility. When white label (Kreyberg) mice are considered it is found that in 16 female mice which bore breast tumours and warts, 62.5 per cent of the warts became malignant within the period of the experiment, whereas in 46 females without breast tumours 69.5 per cent became malignant. Again, from table VIII it is seen that of all the strains malignancy is most likely to supervene in black agouti mice

TABLE VIII.

*Incidence of malignancy (estimated histologically) after 29 weeks of tarring*

Strain	No of mice which developed warts	Mice examined histologically at 29 weeks		Simple warts		Malignancy without invasion		Malignancy with invasion		Malignancy (total)	
		No	Per cent	No	Per cent	No	Per cent	No	Per cent	No	Per cent
IF	219	100	45.7	32	32.0	11	11.0	57	57.0	68	68.0
White label	114	98	86.0	34	34.7	10	10.2	54	55.1	64	65.3
Bagg albino	79	74	93.7	43	58.1	10	13.5	21	28.4	31	41.9
Black agouti	94	86	91.5	19	22.1	7	8.1	60	69.8	67	77.9

*The influence of sex upon the appearance of tar warts (table IX)*

The numbers of mice of each sex used in the various experiments were roughly equal (table IX), except in the case of the Bagg albinos where the proportion of females to males was 83 to 32. Warts appeared approximately at the same time in mice of each sex.



TABLE IX

Influence of sex upon the appearance of tar warts

Strain.	IF				DK				JCTF				White label (Kreberg)				Bagg albino				Black agouti			
	Female		Male		Female		Male		Female		Male		Female		Male		Female		Male		Female		Male	
Week.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.	No. with warts	Per cent.
7-12	20	12.4	40	37.7	3	3.4	6	0.2	1	1.0	12	10.0	0	0	0	0	0	0	0	0	0	0	1	2.0
13-16	41	14.7	31	29.2	20	22.5	22	27.2	18	33.3	22	20.3	5	7.0	7	0.7	1	1.2	2	0.3	3	0.7	6	12.0
17-20	17	11.4	10	17.9	33	37.1	31	38.3	19	35.2	24	32.0	10	20.8	17	23.0	10	12.0	4	12.5	17	37.8	19	38.0
21-29	7	5.9	14	13.2	27	30.3	17	21.0	14	25.9	1	10.0	38	53.5	28	38.0	51	65.1	8	25.0	25	55.6	23	40.0
30*	3	2.5	2	1.9	6	0.7	0	7.1	2	3.7	5	0.7	0	12.7	20	27.8	18	21.7	18	50.3	0	0	1	2.0
Total	118		106		89		81		64		73		71		72		63		12		15		50	

\* TI -- 20 days before warts at the end of 20 weeks of farrowing

In the white label (Kreyberg) strain, rather more male than female mice had failed to develop warts by the end of the experiment (27·8 per cent of males compared with 12·7 per cent of females). Similarly in the Bagg albino mice, 56·3 per cent. of males and 21·7 per cent of females had not developed warts at the end of 29 weeks' tarring. So that there appears to be some slight delay in the development of warts in male mice of these two late strains. The black agouti mice did not show this difference.

*The influence of sex upon the development of malignancy  
(tables X and XI)*

There is no marked difference in the interval between wart appearance and the development of clinical malignancy in male and female mice in either the early strain IF or the late strains white label (Kreyberg) and black agouti (table X). But consider-

TABLE X.

*Comparison of clinical and histological malignancy in males and females.*

Strain	Interval between appearance of wart and clinical malignancy				Histological examination at 29 weeks of tarring					
	Female		Male		Female			Male		
	No of mice	Average interval	No of mice	Average interval	No of mice	Per cent simple	Per cent malignant	No of mice	Per cent simple	Per cent malignant
IF	15	10·0	32	10·3	50	22·0	78·0	50	12·0	58·0
White label (Kreyberg)	28	6·1	15	7·3	51	25·5	75·5	17	11·7	55·3
Black agouti	30	4·6	25	5·8	10	10·0	90·0	16	32·6	67·1

ably more females than males in each strain have become histologically malignant by the end of the experiment (table X). This difference is statistically significant. Similarly, whether the warts had existed for a short time (0·9 weeks) or for a longer time (table XI), in each case a greater proportion of female than male mice bore malignant tumours. Thus in male mice together with a slight delay in the development of warts there is also a tendency for them to take longer to become malignant.

**Experiment V.**

The experiments just described were started before the discovery by Cook and his co-workers (summarised by Cook *et al.*, 1937) of pure carcinogenic substances; recently it was thought desirable to test the mice with 3-1-benzpyrene and methylcholanthrene in addition to tar. At the same time an attempt was made to obtain

TABLE XI  
*Comparison of malignancy according to the time of existence of the wart in males and females*

Strain		Time of existence of wart in weeks															
		0-9						10-11						12-21			
		Female			Male			Female			Male			Female		Male	
		No with warts	Per cent.	No with warts	Per cent.	No with warts	Per cent.	No with warts	Per cent.	No with warts	Per cent.	No with warts	Per cent.	No with warts	Per cent.	No with warts	Per cent.
IF	Simple	3		7		8	50	13	72	11	35	15	56	11	26	8	27
		0		0		8	10	5	28	20	65	12	44	32	74	22	73
	Malignant	10	17	4	50	6	16	14	47	2	18	2	20	0		0	
		2	83	4	50	28	82	10	53	9	82	8	80	1		0	
White label (Kroyberg)	Simple	1	20	5	67	1	5	4	20	2	13	4	29	1		0	
	Malignant	4	80	3	33	20	05	10	80	13	87	10	71	0		3	
Black agouti	Simple																
	Malignant																

a more accurate estimate of the actual onset of malignancy in warts by means of biopsy under anaesthesia

Branch (1936) painted the skin of mice twice weekly with a 0.5 per cent solution of 1,2,5,6-dibenzanthracene in pure benzol and found that almost twice as many malignant tumours developed in a low breast cancer as in a high breast cancer strain, but no such disproportion was observed in injected animals which developed sarcomata. Andervont (1934, 1935) however showed that single injections of the substance produced sarcomata in proportions varying with the strain of mice employed, strains with a high mammary cancer incidence reacting earlier than those with a low, and Boyland and Warren (1937) found that the black agouti strain (CBA) was less susceptible to the carcinogenic action of methylcholanthrene injected *sub cutem* than the Simpson strain of albino mice (high breast cancer incidence).

Application of the carcinogenic substance in 0.3 per cent solution in benzene was made once weekly to the skin of the back between the shoulder blades, without preliminary epilation. As soon as the wart was thought to be malignant, a small piece of the growing edge was removed under ether and urethane anaesthesia and submitted to microscopical examination. If found to be malignant, the mouse was killed, if not malignant, the tumour was observed for a further period and the mouse killed as soon as malignancy was thought to have supervened. By this means it was possible to obtain a fairly accurate estimate of the actual date of onset of malignancy, but it must be remembered that even biopsy may fail to reveal malignancy if the right piece of tissue is not removed.

#### *A Application of methylcholanthrene (0.3 per cent solution in benzene) \**

(1) *Appearance of warts in IF and black agouti mice* Using methylcholanthrene in 0.3 per cent solution, an agent which has

TABLE XII.

*Appearance of methylcholanthrene and 3,4-benzpyrene warts*

Agent	Methylcholanthrene				3,4-Benzpyrene							
Weeks	IF		Black agouti		IF		White label (Kreyberg)		Bagg albino		Black agouti	
	No with warts	Per cent	No with warts	Per cent	No with warts	Per cent	No with warts	Per cent	No with warts	Per cent	No with warts	Per cent
0-12	16	64.0	0	0	1	3.8	1	6.3				
13-16	3	12.0	4	16.7	6	23.1	3	18.8				
17-20	4	16.0	8	33.3	15	57.7	4	25.0			1	5.0
21-30	3*	8.0	12	50.0	4	15.4	8	50.0	1	5.9	3	15.0
31-40									7	41.2	15	75.0
41-50									9	53.0	1	5.0
Total	25		24		26		16		17		20	

\* One of these mice died at 23 weeks without a wart

rather greater carcinogenic power than the tar in use in this laboratory, 16 of 25 IF mice (64 per cent) had developed warts

\* Supplied by Messrs Hoffmann-La Roche

by the twelfth week compared with none of 24 black agouti mice. Half of the black agouti mice developed their warts between 21 and 30 weeks, by which time all the IF mice except two already had warts (table XII). The mortality in this experiment was very low, three IF mice dying at 6, 9 and 23 weeks and two black agouti mice at 14 weeks without warts. Fifty per cent of survivors bore warts at 10½ weeks in the case of IF and at 20 weeks in the case of black agouti mice.

(2) *Development of malignancy* Nineteen warts in the IF mice became malignant, three mice died without developing a wart, in four the wart remained simple until death and in one it regressed. The average interval between the appearance of a wart and the development of malignancy as estimated by biopsy was 11.4 weeks, the range being from 2 to 19 weeks. All the warts (24) in the black agouti mice became malignant but two mice died without warts. The average interval between the appearance of a wart and the development of malignancy was 5.5 weeks, the range being from 1-15 weeks. Thus even though IF warts in general appeared earlier than black agouti warts, they took considerably longer to become malignant.

#### B Application of 3:4 benzpyrene (0.3 per cent in benzene) \*

(1) *Appearance of warts in IF, white label (Kreyberg), Bagg albino and black agouti mice* That this is a much weaker carcinogenic agent than methylecholanthrene or tar is shown by the fact that whereas 64 per cent of IF mice had developed methylecholanthrene warts by the end of the 12th week, only 3.8 per cent had developed benzpyrene warts, the majority of these warts occurred between the 13th and 20th weeks (table XII). All the IF mice had developed warts by the end of the 28th week, whereas the first Bagg albino mouse did not develop a wart until 27 weeks and the majority appeared between the 40th and 40½ weeks. The warts in black agouti mice appeared earlier than in Bagg albino but considerably later than in IF mice. All white label (Kreyberg) mice had developed warts by the end of 25 weeks, the majority appearing between the 21st and 25th weeks. Thus their response approximates more closely to that of IF mice.

Fifty per cent of survivors bore warts at the following times: IF 17½ weeks, white label (Kreyberg) 19 weeks (i.e. actually earlier than with tar), Bagg albino 40½ weeks and black agouti 34 weeks. This method of estimation shows the difference between the early and late strains in a striking way.

(2) *Development of malignancy* It is interesting to note that although the weaker carcinogenic agent delays the development

\* Supplied by Messrs. Light

of warts in IF, Bagg albino and black agouti mice, and accentuates the differences between them, the interval between wart appearance and the development of malignancy is only slightly delayed when compared either with tar or with methylcholanthrene (table XIII). This interval is remarkably consistent when it is considered that in the tar experiments malignancy was estimated clinically, whereas in the later experiments it was estimated by biopsy.

TABLE XIII.

Interval between wart appearance and development of malignancy

Interval between wart appearance and development of carcinoma				Methylcholanthrene.		Benzpyrene	
Strain	No of mice	Tar		No of mice	Average interval (weeks)	No of mice	Average interval (week.)
		No of mice	Average interval (weeks)				
IF	77	10.5	19	11.4	25	12.2	
White label	43	6.5	16	..	14	8.2	
Bagg albino	11	6.9	14	..	20	6.5	
Black agouti	35	5.2	24	..	..	..	

The numbers of mice used in these experiments are much smaller than those in the original tar experiments and the number is low. In general, the results obtained with pure carcinogenic substances confirm those already obtained with tar except that white label (Kreyberg) mice appear to be more sensitive to pyrene than they are to tar. It is not desired to stress this point as the numbers are too small.

The warts induced with methylcholanthrene and benzpyrene have similar histological characters to those induced with tar. In regard to the malignant change there is a point of difference. With tar, the cells of a squamous carcinoma occasionally assume a spindle form, the spindle cells being epithelial in origin. With methylcholanthrene and benzpyrene, the spindle-cell change is very much more frequent and has been observed especially often in black agouti mice (50 per cent of mice). It was observed less frequently in IF and not at all in white label (Kreyberg) or Bagg albino mice.

DISCUSSION.

A successful attempt to breed a strain of mice (IF) specially sensitive to the development of the tar wart has been described. Contrary to expectation, these mice do not show any tendency towards rapid development of malignancy. In these respects they differ from the high breast cancer strain described by Reinhard and

Candee In 1934 Kreyberg described an experiment in which a series of mice (red label) also developed benign tar tumours early and he concluded that in this respect they were genetically different from a strain (white label) which developed warts considerably later. No information was given as to the relative dates of development of malignant tumours.

When the IF females are considered separately, there is no significant difference between them and the males in respect of the date of appearance of warts (table IX) but a greater number of females than males had become malignant by the end of the experiment (table X). Kreyberg (1935) and Reinhard and Candee also found that there was a delay in the appearance of tar cancers in males as compared with females. I have found a much less significant delay in male mice of Kreyberg's strain with the highly carcinogenic tar in use in this laboratory.

The response of IF mice to the application to the skin of two pure carcinogenic substances, 3,4-benzpyrene and methylcholanthrene, is similar to the response to tar. Although the appearance of warts could be considerably delayed by the use of a weak carcinogenic agent such as 0.3 per cent 3,4-benzpyrene in benzene, the supervention of malignancy was only slightly delayed (tables XII and XIII). Their response to other types of carcinogenic stimulus is not yet known. No spontaneous breast tumour has been observed among them, although no attempt has been made to observe the females into old age. There is much individual variation in the date of appearance of warts and in the development of malignancy (tables I and XII).

The response of other inbred strains to tar and pure carcinogenic substances has also been investigated. It seems clear that this has no actual relation to the tendency to develop spontaneous breast cancer, for a late tar response was given by strains with both high and low incidence of mammary cancer. Little's line of Bagg albinos, having a high incidence of mammary cancer, gave the most delayed tar response and the presence of a breast tumour in individual mice did not delay the appearance of tar warts. In reviewing the information available on this subject, there is great variety in the results obtained in different laboratories. For example Kreyberg (1935), Lynch (1933-34) and Branch found that lines with a low incidence of spontaneous breast cancer were more susceptible to induced skin cancer than lines free from breast cancer. By contrast, Reinhard and Candee found that the opposite was the case. Also Andervent (1935) and Beyland and Warren found that high breast cancer strains were more susceptible (as shown by the development of sarcomata) to the injection of 1,2,5,6-dibenzanthracene and methylcholanthrene respectively than low breast cancer strains. The conclusion to be drawn from

these observations appears to be that each strain has its own type of response to any one carcinogenic agent. As for the reaction of the skin is concerned, its sensitivity appears to be independent of coat colour.

Inbreeding for many generations does not diminish hereditary variability in the date of appearance of the wart or in the interval between wart appearance and the development of malignancy. One strain, black agouti (CBA), inbred for 30-32 generations, has less individual variation than the others (table V) and also a pronounced tendency to the rapid development of malignancy (table VI-VII, VIII and XII).

The significance of this work in relation to the development of skin cancer in man is of importance. Much more information is required before suggestions other than tentative can be made. However, it may be pointed out that a general tendency to the development of benign tumours is not necessarily associated with rapid development of malignancy (strain II) and that delay in the development of benign tumours can be associated with rapid development of malignancy (strain black agouti CBA). The whole of the evidence points to the conclusion that the genetic factor cannot be ignored.

#### CONCLUSIONS

##### 1. Coat colour

Coat colour is not a factor in the development of skin cancer in the mouse. The response to carcinogenic agents is determined by other factors.

##### 2. Inbreeding

Inbreeding for many generations does not diminish hereditary variability in the date of appearance of the wart or in the interval between wart appearance and the development of malignancy.

##### 3. Strains

There are marked differences in the response of the strains to the various carcinogenic agents. The black agouti (CBA) strain has a pronounced tendency to the rapid development of malignancy.

##### 4. Benign tumours

A general tendency to the development of benign tumours is not necessarily associated with rapid development of malignancy. Delay in the development of benign tumours can be associated with rapid development of malignancy.

##### 5. Genetic factor

The whole of the evidence points to the conclusion that the genetic factor cannot be ignored in the development of skin cancer in the mouse.



(6) The results of applying methylcholanthrene to the skin of mice of IF and black agouti strains are similar to those obtained with tar. When 3,4-benzpyrene is used in IF, white label (Kreyberg), Bagg albino and black agouti mice, the results are also similar except in the case of the white label mice, which seem to be particularly sensitive to benzpyrene. The weaker carcinogenic agent serves to accentuate the differences between early and late strains in respect of wart development but not in respect of development of malignancy.

(7) The mice of the IF strain have not been observed to develop spontaneous breast cancer.

(8) The genetic constitution of the mouse rather than the actual development of a spontaneous breast tumour is the factor concerned in the delay in development of the tar warts.

(9) Numerous attempts to breed a strain of mice specially resistant to the action of tar upon the skin were unsuccessful.

(10) Prolonged inbreeding does not decrease the range of date of wart development compared with stock mice. In other words, there is great individual variation within the strain in response to carcinogenic agents applied to the skin.

I wish to express my thanks to Professor R. D. Passoy at whose suggestion these experiments were undertaken and to Dr J. C. Mottram and Professor F. A. E. Crow for helpful suggestions. To my colleagues in this Department I am grateful for criticism and suggestions. I have great pleasure in acknowledging the invaluable assistance of Dr M. Young in the statistical analysis of the results.

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## A SPECIAL FORM OF ERYTHROCYTE POSSESSING INCREASED RESISTANCE TO HYPOTONIC SALINE

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(PLATES LXIII AND LXIV)

ALTHOUGH human erythrocytes in stained films usually show a relatively pale centre and a more deeply stained outer zone, there is another form in which both the centre and the periphery are deeply stained and a pale or unstained intermediate zone lies between them, giving an appearance somewhat like the alternate dark and light concentric zones of a target (fig 1). So far as I am aware this form of red corpuscle has previously received little attention. Since the completion of the greater part of the present work Haden and Evans (1937) have described this form as a characteristic feature of sickle cell anemia. They refer to them as "dimpled corpuscles," and liken their form to that of a Mexican hat, but, for reasons presently to be given, "dimpled" appears to be an inaccurate description, and the term "Mexican hat corpuscles," though picturesque, is unwieldy, even if it represents the true form of the corpuscles. Therefore, with apologies for the introduction of a new term, they will here be called "target types of red blood corpuscle," or more briefly, "target corpuscles." I have deliberately chosen a name which refers only to their appearance in stained films and not to their three dimensional form.

Concerning this target type of corpuscle Haden and Evans (p 142) say "This unusual shape must be related in some way to the abnormal tendency to hemolysis in sickle cell anemia and may determine the ease of fragmentation. One occasionally sees a few cells of this shape in other anemias, but they are never present in any significant number except in sickle cell anemia. They were constant in all the cases in which we have made an examination." It is convenient to defer discussion of these conclusions, but it is necessary to say at once that Haden and Evans are mistaken in supposing that target corpuscles are "never present in any significant number except in sickle cell anemia." They are abundant, for instance, in many cases of obstructive jaundice. Fig 1 is a photograph of the blood film of such a patient,

an English lady aged 68 who was suffering from an obstructive jaundice apparently due to carcinoma of the head of the pancreas and in whom there was no reason whatever to suspect sickle-cell anaemia.

In obstructive jaundice some degree of anaemia is common, particularly if the jaundice is long-standing; but even when the haemoglobin content and blood count are normal, and when there is no other abnormality in the film, target corpuscles are often conspicuous and abundant. Now it has long been known that increased resistance to hypotonic saline is often a feature of the red corpuscles in obstructive jaundice (von Limbeck, 1896; McNeil, 1910-11, Meulengracht, 1922; and others), and hence it seemed possible that there might be some relationship between the target type of red corpuscle and increased resistance to hypotonic saline.

In order to test this possibility I determined in each of 50 patients (1) the percentage of the red cells which were not haemolysed by 0.30 per cent. NaCl solution; (2) the presence or absence of target corpuscles in stained films. To avoid unnecessary repetition, corpuscles resistant to 0.30 per cent. NaCl solution will now be referred to simply as "resistant" corpuscles, and the term "resistance" will be used only in the limited sense of resistance to hypotonic saline. The patients were selected so that there were approximately equal numbers with and without target corpuscles; such corpuscles were searched for in every blood film examined—not only in cases of jaundice—and alternate cases in which they were present and in which they were absent were included in this series. The results of these investigations are shown in table I.

### METHODS

#### (1) *Determination of percentage of "resistant" corpuscles.*

A 0.30 per cent. NaCl solution was prepared by appropriate dilution of a stock 1 per cent. solution of sodium chloride which had been prepared from pure dried NaCl and checked by titration with standard silver nitrate solution. To determine the percentage of "resistant" corpuscles, two red cell counts were made, one using the ordinary isotonic diluting fluid (0.1 per cent. gentian violet in 0.9 per cent. NaCl solution), the other using 0.30 per cent. NaCl solution as the diluting fluid. A Barker counting chamber was used and the total area counted in each case was such that it included not less than 100 corpuscles. The first count represented the total number of red corpuscles per cmm., the second the total number of "resistant" corpuscles per cmm. The latter number was then expressed as a percentage of the former.

As far as possible only completely unhaemolysed corpuscles were included in the second count. A certain very variable number of "ghost" cells were often visible in the 0.30 per cent. NaCl solution, and although it was usually easy to distinguish them from the unhaemolysed cells, occasionally it was difficult. The use of capillary blood introduced another variable factor, as Winthby and Hynes (1935) have shown that erythrocyte resistance is affected by the degree of oxygenation of the blood, which may not be

constant in different samples obtained by ear prick. The possible errors due to these causes were not controlled, but in order to obtain some idea of their magnitude ten separate determinations of the percentage of "resistant" corpuscles were made on the same patient (147/37) during a period of 26 hours. The results were 4.2, 5.7, 5.3, 5.7, 5.4, 4.8, 4.7, 4.5, 4.9, 5.1. Such variations could hardly affect the results given below.

(2) *Determination of presence or absence of target corpuscles*

Blood films were made on slides, dried and stained for 3 minutes with Jenner's stain. Counterstaining with dilute aqueous eosin makes the target corpuscles more conspicuous, but it is not necessary and was not used during this investigation. The presence or absence of target corpuscles was determined in the following way. They were said to be absent unless two or more were found during three minutes search of a satisfactory film. This criterion does not pretend to exclude absolutely the presence of target cells; it was only necessary that the criterion employed should be a constant one. Often the frequency of target cells appeared to be affected by the thickness or thinness of the film. This phenomenon was of little importance in the first investigation (table I), in which the percentage of target corpuscles was not determined. In the second investigation (table II), in which the percentage of target corpuscles was determined, care was taken that all films were of even thickness.

## RESULTS

The results given in table I strongly suggest that there is a relationship between the presence of target corpuscles and resistance to hypotonic saline. It will be seen that there are 20 cases altogether in which more than 2.0 per cent of the corpuscles were resistant to 0.30 per cent NaCl solution, and in every one of these target corpuscles were present, whereas of the 21 cases with less than 0.5 per cent of "resistant" corpuscles, only one showed

TABLE I

*The percentage of red corpuscles resistant to 0.30 per cent NaCl solution, and the occurrence of target corpuscles, in fifty selected cases*

Percentage of "resistant" corpuscles	Number of cases	Target corpuscles present	Target corpuscles absent
Less than 0.05	4	0	4
0.05-0.1	6	0	0
0.1-0.25	5	0	5
0.25-0.50	6	1	5
0.5-2.0	9	5	4
2-5	5	5	0
5-10	6	6	0
10-20	5	5	0
20-30	1	1	0
30-40	3	3	0
Totals	50	20	24

target corpuscles. Further, although no attempt was made to demonstrate a quantitative relationship, it was noticed that target cells were always very abundant in cases with the highest percentages of "resistant" corpuscles.

In order to obtain additional evidence concerning this relationship, observations were made on a few patients in whom an alteration in the corpuscular resistance was likely to take place within a comparatively short period of time. It has been shown by many workers (*e.g.* Pel, 1912; Pearce, Krumbhaar and Frazier, 1918, Whitby and Hynes, 1935) that in animals and in man splenectomy is usually followed (except, possibly, in acholuric jaundice) by an increase in the corpuscular resistance. It seemed possible therefore that, if repeated observations were made on patients submitted to splenectomy for reasons other than acholuric jaundice, an increase in the percentage of "resistant" corpuscles might be observed, and this might or might not be associated with the appearance of or with increased frequency of target corpuscles in stained films. The results are shown in table II.

TABLE II

*The corpuscular resistance and the frequency of target corpuscles after splenectomy.*

Case no	Diagnosis	Date	Time after operation (days)	Red cell count (millions per c mm)	Colour index	"Resistant" corpuscles (per cent)	Target corpuscles (per cent)
18 37	Thrombocytopenic purpura splenectomy 9 2 37	10 2 37	1	4 81	0 83	0	0
		11 2 37	2		.	0	0
		15 2 37	6	3 33		1 4	0 1
		18 2 37	9	3 49	0 88	2 75	0 8
		22 2 37	13	3 71	0 85	3 0	0 2
		20 3 37	39	3 92	0 83	5 4	1 8
		7 1 37	57	4 74	0 84	6 1	1 9
22 37	"Splenic anaemia" splenectomy 24 2 37	24 2 37	0	4 31	0 87	0 02	0
		12 3 37	16	4 84	0 90	1 4	0 2
		28 5 37	77	4 71	0 95	4 8	2 3
63 37	Thrombocytopenic purpura splenectomy 4 6 37	5 6 37	1	4 50		0 27	0
		11 6 37	7	4 40	0 82	1 3	1 9
		19 6 37	15	4 40	0 80	1 6	1 1
		22 9 37	110	5 20	0 81	13 7	1 9

In determining the percentage of target corpuscles 1000 red cells were counted. These results provide further evidence of an association between the two characteristics under discussion, although in at least the third case (63 37) the relationship is apparently not a quantitative one. Nevertheless in each of these three cases target corpuscles were absent at the time of the first observation, in each an increase in percentage of resistant corpuscles was associated with the appearance of corpuscles of the target



### PLATE LXIII

FIG 1—Stained dried blood-film of a patient (5537) with obstructive jaundice showing numerous target corpuscles. In 0.30 per cent NaCl solution 33 per cent of the red corpuscles remained unhemolysed.

FIG 2—Expt I stained film of untreated blood.

FIG 3—Expt I isolated "resistant" corpuscles of the blood shown in fig 2.

FIG 4—Wet unstained preparation of the blood of a patient with obstructive jaundice whose blood film showed many target corpuscles.

FIG 5—Control preparation of normal blood treated in the same way as the blood in fig 4.



A. C. PATTERSON'S METHOD

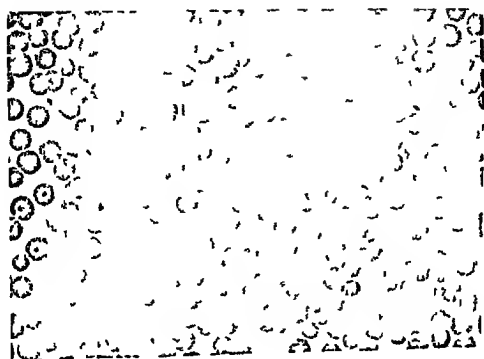


FIG. 1

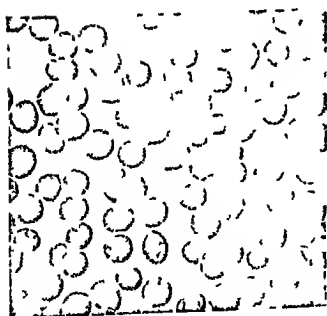


FIG. 2

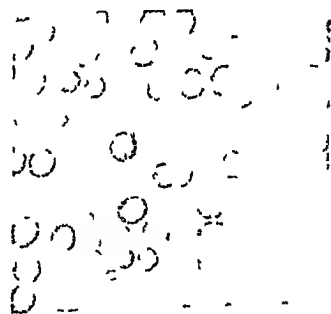


FIG. 3

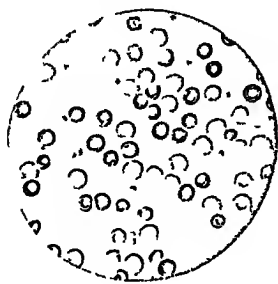


FIG. 4

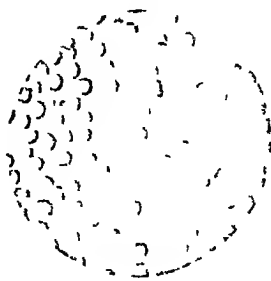


FIG. 5



type, and in the first two cases, except in two observations, the target corpuscles became more abundant as the "resistant" corpuscles increased in number.

It has already been mentioned that the increased corpuscular resistance in many cases of jaundice is associated with the presence of target corpuscles. If the jaundice disappears, the corpuscular resistance soon returns to normal, and in the few cases that I have examined the target corpuscles have disappeared at the same time.

The question next arises whether the target corpuscles are themselves abnormally resistant, or whether, owing perhaps to a common cause, their presence is merely associated with the presence of other corpuscles whose resistance is increased. In order to decide this the following experiment was made.

#### Experiment I

##### *Isolation and examination of red corpuscles resistant to 0.30 per cent sodium chloride solution*

By venepuncture 5 c.c. of venous blood were obtained from a patient with obstructive jaundice whose blood film showed many target corpuscles, heparinized, and oxygenated by shaking. Several blood films were then made, after which the blood was centrifuged and the plasma removed as completely as possible by means of a pipette. The plasma was preserved. About 0.5 c.c. of the deposit of red corpuscles was added to 100 c.c. of 0.30 per cent NaCl solution, shaken gently and allowed to stand for 1½ hours at room temperature, it was then centrifuged and the deposit resuspended in normal isotonic saline. After standing for ½ hour this was again centrifuged. The deposit showed two clearly defined layers: the upper bright pink and semi-translucent, the lower dark red and opaque. The supernatant fluid and as much as possible of the upper layer of the deposit were removed with a pipette and replaced by some of the original plasma, so that there were about 4 volumes of plasma to one of corpuscles. After this had been mixed and had stood for 10 minutes the plasma was removed by centrifuging once more and the same quantity of the original plasma again added. In this way a suspension was obtained, in their own heparinized plasma, of those cells which were resistant to 0.30 per cent NaCl solution. Films were made from this suspension and compared with those made previously from the whole blood.

It was obvious that there was a much higher proportion of target corpuscles among the "resistant" cells than in the whole blood (compare figs 2 and 3). Ghost cells and cells apparently showing all degrees of hæmolysis were present among the "resistant" cells, but 58.6 per cent of the completely un hæmolysed cells were definite target corpuscles compared with 6.9 per cent in the whole blood, 1000 cells being counted in each case.

In order to confirm this result the experiment was repeated upon blood from another patient with obstructive jaundice. This time, besides the sample of blood that was washed with 0.30 per cent NaCl solution, a second sample was treated with 0.25 per cent

solution in order to ascertain whether the proportion of target corpuscles was still higher among the cells resistant to this dilution, while yet a third sample was treated in exactly the same way, except that isotonic (0.9 per cent) saline was used (table III). The third (control) sample was intended to ensure that the increase in the number of target forms was not due merely to the effects of the washing, centrifuging, etc.

TABLE III.  
*Results of expt. I.*

Nature of specimen	Target corpuscles (per cent)
Untreated blood	3.8
Corpuscles washed in 0.9 per cent NaCl sol	5.2
Corpuscles resistant to 0.30 per cent NaCl sol	56.6
Corpuscles resistant to 0.25 per cent NaCl sol	63.2

In determining the percentage of target corpuscles, 1000 cells were counted in each case and only completely unhaemolysed cells were included. Since, however, it was sometimes difficult to decide whether a corpuscle was completely unhaemolysed or not, and since the proportion of target cells appeared to vary somewhat in different areas of the films, I do not regard the difference between 56.6 and 63.2 per cent as significant. If there are more target corpuscles among the cells resistant to 0.25 per cent NaCl solution than among the cells resistant to 0.30 per cent, the difference cannot be very great. On the other hand there are undoubtedly many more corpuscles of the target type among the "resistant" cells than among the cells of the whole blood.

It appears, therefore, that target corpuscles are not only associated with an increased proportion of "resistant" cells, but are themselves more resistant than the cell population as a whole.

#### *Other characteristics of the target type of red corpuscle.*

In view of the apparent significance of the target corpuscles it seemed worth while to investigate their characteristics more closely. Further examination of stained dried films suggests that target cells tend to be both slightly smaller in diameter and more perfectly round than the rest of the corpuscles. The latter feature is only a general impression, but the former is supported by measurements. In a blood film from a case of obstructive jaundice, the mean diameter of 500 red corpuscles selected at random (including 4.0 per cent. of target corpuscles) was  $8.045 \mu$ , standard deviation  $0.650 \mu$ , whereas the mean diameter of 500 target corpuscles in the same film was  $7.662 \mu$ , standard deviation  $0.677 \mu$ .

The difference between the two means is 3.88 times the standard error, so that it may reasonably be regarded as significant.

The appearance of the target forms in dried films suggests that instead of being simple bi-concave discs they have a central thickening which would entitle them to be described as "umbonate". If a stained dried film containing this form of corpuscle be examined, unmounted, with a binocular microscope and a one sixth objective (not an immersion lens) the stained rims and centres of the target forms do in fact appear to correspond to definite elevations of the surface. The appearance is shown well in the bas-relief photograph of Haden and Evans (fig. 3), who refer to the "central, sugar leaf elevation" and liken the cross section of these corpuscles to that of a Mexican hat. They also refer to them as dimpled corpuscles, but since the term "dimple" is commonly used to denote a small depression rather than a projection, "umbonate" corpuscles would seem to be a better description.

It is necessary, however, to consider the possibility that this umbonate form is only acquired during the preparation of the dried film, especially as it has already been mentioned that the frequency of target corpuscles often appears to vary more in different parts of the same film than is likely to be due to chance alone, and that it is apparently affected by the thickness of the film. Expt. II was designed to settle this point.

### Experiment II

#### *Observations on wet unstained preparations—comparison between such preparations and dried films*

Dried films and fresh wet preparations were made from the heparinised venous blood of a patient (147/37) with obstructive jaundice. In making the wet preparations the blood was diluted in a blood-count pipette with about 100 volumes of plasma obtained from another sample of the same blood. This dilution was necessary because otherwise the corpuscles were too crowded. The dried films, unstained and unmounted, showed frequent corpuscles with the target appearance when examined with a  $\frac{1}{4}$  in. objective, but only very few rather indefinite ones were found in the wet preparations. One of the dried films was fixed by immersion in methyl alcohol for 30 seconds, and after being dried once more a drop of plasma was placed upon it and a coverslip applied. This preparation now differed from the fresh wet preparations only in that the corpuscles had been dried upon the slide and fixed, but it still showed frequent target corpuscles, whereas no definite target cells could be seen in the unfixed preparations. Thus the fresh blood must have contained corpuscles which only acquired the target appearance during the preparation and drying of the films.

Now there were in the wet preparations corpuscles different from any that were seen in control preparations of normal blood (compare figs. 4 and 5), and it was natural to wonder whether these might be the corpuscles which became target cells in dried films. When resting upon the slide they were seen as rings: the ring appeared denser than an ordinary corpuscle and was limited by an inner boundary almost as sharp as the outer border of the cell, while the central area enclosed by the ring appeared almost or quite empty (fig. 4). The explanation of this appearance became clear when the corpuscles were set in motion by touching the cover glass. Many then floated and rolled over so that they could be seen from all aspects. It could then be seen that the corpuscles which, when they had settled upon the slide, had a ring-like appearance were in reality bowl-shaped. In fig. 4 one such corpuscle on its side and two beside it almost completely on their sides can be seen above the centre, while others are seen in intermediate positions. When such a bowl-shaped corpuscle lies upon its bottom or its rim and is viewed from above, transmitted light meets much more tissue in the approximately vertical sides of the bowl than it does in the centre. Consequently the sides appear denser and give rise to the ring-like appearance described above. A model of such a bowl-shaped corpuscle was made with plasticine (figs. 6a and 6b), and, since plasticine is not transparent to light, an X-ray photograph was taken (fig. 6c) which shows a ring-like appearance similar to that observed in the fresh unstained preparations. In the control preparations of normal blood some of the corpuscles were concavo-convex instead of bi-concave, and the bowl-shaped corpuscles appear merely to represent an exaggeration of this form. If normal blood be diluted with 0.9 per cent. sodium chloride solution, often many of the corpuscles become definitely bowl-shaped—at least temporarily.

The scarcity of target corpuscles in wet unstained preparations makes it difficult to determine the three-dimensional form of such corpuscles, because it is only in wet preparations that the cells can be set in motion and observed from all aspects. In dried films they can only be observed from one aspect, which is not sufficient to enable their three-dimensional form to be ascertained. If the bas-relief photograph of Haden and Evans (fig. 3) is in fact of a fresh wet preparation, as it appears to be, it seems that corpuscles with the target appearance are sometimes abundant even in such preparations, but this has not been my experience. Occasionally I have seen definite target cells in wet unstained preparations of cells suspended in their own plasma, but such target corpuscles are usually so adherent to the slide that they remain still when most of the other corpuscles are set in motion by touching the



## PLATE LXIV

FIG. 6 —Plasticine model of bowl shaped corpuscle (a) the entire model, (b) the model bisected, (c) X-ray photograph of the entire model

FIG. 7 —Plasticine model of a target corpuscle as seen in expt. III (a) the entire model; (b) the model bisected, (c) X-ray photograph of the entire model







coverglass During attempts to obtain microscopic sections of target corpuscles, however, it was found that if the cells are not suspended in their own plasma but in formal saline solution, cells with the target appearance may be seen floating free and their form can then be ascertained

### Experiment III

#### *Observations on corpuscles suspended in formal-saline solution*

Another sample of the same blood that was used in expt II was mixed with about 20 times its volume of formal saline solution (4 per cent formaldehyde in 0.9 per cent NaCl solution) and allowed to stand for several hours. As much as possible of the supernatant fluid was then removed with a pipette. Wet preparations of the deposit were made as in expt II except that formal saline was used as the diluent instead of plasma. Other preparations were made using a saturated solution of eosin in formal-saline as the diluent in order to stain the corpuscles. There were frequent target corpuscles in these preparations. Each time a target corpuscle was observed an attempt was made to set it in motion by touching the coverglass. Frequently this attempt was unsuccessful, and it became clear that the target corpuscles were much more often adherent to the slide than were the other corpuscles. It was usually impossible to dislodge those corpuscles in which the target appearance was most conspicuous, but others which undoubtedly had the target appearance, although the intermediate zone was not so pale as usual, could frequently be dislodged. Many such corpuscles were observed from all aspects as they turned over and over, and in this way it was possible to determine that their form was like that of the plasticine model shown in figs 7a and 7b. At first sight it may not be obvious why a transparent body of approximately this shape should have the target appearance when viewed by transmitted light, but an X ray photograph of the model (fig 7c) closely resembles the appearance of a target corpuscle.

The results of expts II and III suggest that in fresh blood "resistant" corpuscles tend to be bowl shaped, and that in certain circumstances such as the preparation and drying of films and exposure to formal saline the bowl shaped corpuscles acquire the target appearance by changing their shape. The exact shape of the fully developed target corpuscles could not be determined because they were always adherent to the slide, but the shape illustrated in figs 7a and 7b appears to be a sub terminal stage. Corpuscles with this shape did have a definite target appearance and only differed from the target corpuscles seen in films in that their intermediate zone was less pale. An attempt was made to

observe the transformation of bowl-shaped corpuscles into target corpuscles by watching under the microscope freshly spread films while they dried. This was unsuccessful, because if the films were sufficiently thin for the corpuscles to be separated, target cells were present as soon as the films were examined, although the films were still wet. Thus no conclusive proof was obtained that the target corpuscles seen in dried films are in fact derived from the bowl-shaped corpuscles of fresh blood.

*Red cell dimensions in conditions in which target corpuscles are abundant.*

A decrease in thickness appears to be the most constant characteristic of the red cell dimensions in these conditions. This thinness of the corpuscles is not infrequently suggested by their appearance in stained films. They are paler than the colour index would lead one to expect, and occasionally one finds a corpuscle which is obviously folded upon itself or which is completely penetrated by one or more small punched-out holes. These latter abnormalities are presumably artefacts, but they are suggestive nevertheless, as they would be unlikely to occur unless the cells were thin. In fresh preparations it is possible to view the corpuscles from their sides; their thinness can then usually be recognised. This thinness was a very noticeable characteristic in the fresh preparations of the isolated resistant cells of expt. I.

There does not appear to be any completely satisfactory method of measuring the mean corpuscular thickness. It may be calculated from the mean corpuscular diameter and the mean corpuscular volume, assuming the corpuscles to have the form of short cylinders, but the result so obtained can only be regarded as approximate. The imperfections of this method are recognised by Price-Jones, Vaughan, and Goddard (1935), who nevertheless consider it to be of some value and who give the results obtained in 100 normal individuals. Table IV gives the results obtained, using the same technique as these authors, in 9 cases in which target corpuscles were a conspicuous feature of the blood film. It will be seen that the values obtained for the mean corpuscular thickness are definitely low. Although there are three cases in which the mean thickness is just within the statistical limits of normal given by Price-Jones, Vaughan and Goddard, in two of these cases the cells are abnormally large in diameter: so that here also the shape of the cells appears to be altered—the thickness is diminished relative to the diameter, although absolutely it is within normal limits.

von Borek (1926a) and Jørgensen and Warburg (1927) have devised methods which are intended to indicate the relative thickness of the corpuscles as distinct from their absolute thickness. The results obtained by their methods are included in table IV. In

TABLE IV  
*Red cell dimensions in nine cases in which target corpuscles were numerous*

Case no.	Diagnosis.	Red cell count (millions per c. mm.)	Colour index	Target corpuscles (per cent.)	Mean corpuscular volume (cu $\mu$ )	Mean corpuscular diameter ( $\mu$ )	Mean corpuscular thickness ( $\mu$ )	Thickness index (on Moraw)	Calculated mean diameter (Jorgensen and Wadding)
	Normal limits { upper (Price-Juana, Vaughan & Goddard) lower				96.096 75.744	7.686 6.651	2.546 1.729		
1/37	Obstructive jaundice carcinoma of pancreas	4.23	0.83	4.0	87	8.04	1.07	0.81	7.2
89/37	Obstructive jaundice carcinoma of pancreas	3.96	0.96	11.2	90	8.63	1.38	0.71	7.3
69/37	Obstructive jaundice carcinoma of pancreas	4.61	0.97	1.6	97	8.21	1.74	0.81	7.4
83/37	Catarrhal jaundice	5.08	0.86	0.2	77	8.39	1.39	0.61	6.9
31/37	Obstructive jaundice gallstones hypochromic anaemia	4.40	0.54	1.0	66	6.81	1.81	1.62	6.6
67a/36	Jaundice fibrosis of liver	5.03	0.51	6.1	69	8.16	1.22	0.63	6.6
6/36	Splenectomy fibrosis of liver (no clinical jaundice)	6.69	0.7-	0.2	68	7.41	1.57	0.81	6.6
36a	Post gastrectomy anaemia (hypochromic type)	4.70	0.41	0.6	60	7.29	1.47	0.79	6.4
23/37	Hypochromic atrophic anaemia purpura macrocytic anaemia	2.71	0.90	0.8	100	8.14	1.78	0.81	7.6

each case the calculation has been modified by substituting the normal mean values obtained by Price-Jones, Vaughan and Goddard, whose methods I used, for those given in the original descriptions, which were based on different methods of measurement. von Boros found that in 22 normal individuals, his "thickness-index" varied from 1.03 down to 0.94 and he considers that lower values indicate that the cells are thinner or flatter than normal. In Jørgensen and Warburg's method the calculated mean diameter (based on the mean corpuscular volume, assuming the cells to be of normal shape) should be within  $0.4\mu$  of the measured mean diameter. If it is smaller than the measured mean diameter by at least  $0.4\mu$ , then the cells are thinner than normal. Table IV shows that, according to the methods both of von Boros and of Jørgensen and Warburg, the cells are relatively thinner than normal in 8 out of the 9 cases.

*The conditions in which target corpuscles occur*

A further study is being made concerning the conditions in which target corpuscles occur. So far I have not found them in normal individuals, but only 8 normals have been specially investigated. By searching for target corpuscles as a routine in every blood film examined I have found them in 97 cases, of which 85 fall into one or more of the following groups.—

- (1) Jaundice (27 cases)
- (2) A "hypochromic" group consisting of cases in which the colour index was not more than about 0.8 (45 cases)
- (3) Splenectomy (8 cases)
- (4) Steatorrhœa (9 cases)

DISCUSSION.

Haden and Evans observed that target corpuscles were abundant in the blood films of patients with sickle-cell anæmia. They considered such corpuscles to be characteristic of this type of anæmia and to be related to the abnormal tendency to hæmolysis. In the light of the results of the present investigation it will be seen that their observation is susceptible of a different interpretation. Both patients described in their paper had previously undergone splenectomy, both had a hypochromic anæmia and both did in fact show increased resistance to hypotonic saline. The absence of target corpuscles would therefore be more surprising than their presence, and there is no reason to suppose that the form of these corpuscles has anything to do with the abnormal tendency to hæmolysis in sickle-cell anæmia. Presumably some of the other cases of sickle-cell anæmia in which Haden and Evans found target corpuscles had not undergone splenectomy, but even so the presence

of target corpuscles is not surprising, because increased resistance to hypotonic saline appears to be a common characteristic of sickle-cell anaemia apart from splenectomy (Anderson and Wart, 1932; Graham, 1932; Daland and Worthley, 1934-35). This may be a result of the fibrosis and atrophy of the spleen which numerous authors, including Haden and Evans, have described in this disease, and which would be expected to have results similar to those of splenectomy.

The apparently constant association of increased resistance with the target type of corpuscle furnishes strong evidence that the resistance of the corpuscles to hypotonic saline is related to their morphology. The association in acholuric jaundice of peculiarities in red cell morphology with increased fragility had previously suggested such a relationship, but it has been impossible to prove conclusively that these peculiarities are not independent characteristics of that one disease. Abnormally resistant corpuscles, however, occur in conditions so diverse as obstructive jaundice, splenectomy, steatorrhoea, and any of the various conditions which may be associated with a low colour index, in all these, target cells can also be found in the blood film. Further, expt I provides direct evidence that corpuscles of the target type are, in fact, more resistant than the remainder of the corpuscles.

Next it is necessary to consider the mechanism of this relationship. Why do corpuscles of the target type possess increased resistance and why is the increased fragility of acholuric jaundice associated with the presence of the peculiarly dense "microcytes" or "spherocytes"? The most notable attempt to correlate the fragility of erythrocytes with their shape is that of Haden (1931). His theory is essentially mechanical: it maintains that hemolysis will begin when the passage of fluid into the cell due to osmosis creates a certain tension in the cell envelope and that the amount of fluid which can pass in before this tension is created will depend upon the original shape of the corpuscle. To explain his theory it is helpful to compare corpuscles of different shapes with bladders filled to varying amounts with water. A spherical cell will correspond to a bladder which is already completely full, so that no more fluid can pass into it without increasing the tension of the envelope. At the other extreme, a cell which has the form of a thin disc will represent a bladder which is collapsed and almost empty: as fluid passes into such a cell, it will gradually become distended, i.e. its diameter will decrease and its thickness increase and it will become more and more nearly spherical, but there will be no appreciable increase in the tension of the cell envelope until the spherical stage is reached. According to this theory, therefore, a spherical cell will be less resistant to hypotonic saline than a flat cell, and in general the thicker the cell the greater will be the

fragility, the thinner the cell the greater the resistance. It is necessary to emphasise that it is not the absolute thickness but the thickness relative to the size of the cell which should determine the resistance. A cell  $5\mu$  in diameter and  $1\mu$  thick should have the same resistance as one  $10\mu$  in diameter and  $2\mu$  thick, although, absolutely, one is twice as thick as the other.

The evidence in favour of this theory is considerable. Haden found that normal human red corpuscles did in fact become more and more spherical when placed in increasingly hypotonic saline solutions and that the fragility in different species of mammals did appear to be related to the shape of the corpuscles in accordance with the theory. There seems to be no doubt that in acholuric jaundice the corpuscles are thicker or more spherical than normal (von Boros 1926b, Naegeli 1931, Vaughan and Goddard, 1931; Vaughan, 1937). Further, Haden states that in obstructive jaundice, where the resistance is often much increased, the cells are thinner or flatter, than normal. This is supported by Jørgensen and Warburg, who review the literature concerning the diameter of the red corpuscles in jaundice and add data of their own.

There are some observations which appear to be directly opposed to Haden's theory, but none of them are beyond criticism on technical grounds. von Boros (1926b) used his "thickness index" as an indication of the form of the corpuscles and found no relationship between this and the resistance, but as Jørgensen and Warburg point out, the calculation of von Boros' "thickness index" involves certain generalisations concerning the ratio of the diameter of the corpuscles to their volume, which are by no means certainly true. The same applies to all observations which involve the calculation of the mean corpuscular thickness on the assumption that the cells have the form of short cylinders. Consequently, although Haden and Evans found in the two cases which they describe that the fragility was decreased but the corpuscular thickness and the thickness index were normal, this is not conclusive evidence against Haden's mechanical theory. Even the haematocrit method of determining the mean corpuscular volume may not be trustworthy if the corpuscles are of different shapes, for then it is possible that they will pack differently. At present, therefore no conclusive test of the accuracy of Haden's theory is possible.

The results of the present investigation support Haden's theory in its essentials. The corpuscular measurements recorded in table IV are in agreement with that theory, because those cases in which there were numerous target corpuscles also showed an increased percentage of "resistant" corpuscles, and according to Haden's theory the greater the resistance the flatter or thinner the corpuscle, so that in these cases we should expect the mean



corpuscular thickness to be diminished, which in fact it appeared to be. Clearly, however, it can only be partially true that the thinner the corpuscles the greater the resistance, because this takes no account of the target corpuscles. Haden's theory cannot explain why many of the more resistant corpuscles should be of the target type, but it needs only slight modification in order to explain why target corpuscles have increased resistance. Reduced to its essentials, the theory implies that the resistance of a corpuscle is normally determined by the magnitude of its surface as compared with its volume, i.e. by the size of the cell envelope as compared with the size of the whole cell. If the cell envelope is small as compared with the volume of the cell, as in a spherical cell, the resistance is decreased, but if the cell envelope (or surface) is relatively large, as in a flat cell, the resistance is increased. Now target corpuscles in dried films appear to be derived from bowl-shaped corpuscles in fresh blood, and a bowl shaped corpuscle has a greater surface/volume ratio than a disc shaped corpuscle with the same volume and diameter. This becomes obvious if one imagines an attempt to flatten out a bowl shaped corpuscle so that it becomes disc-shaped. Clearly the cell envelope would be thrown into folds, showing it to be larger than that of a disc shaped corpuscle. Indeed, whatever may be the exact shape of the target corpuscles in stained films, it seems clear that owing to the undulations of their surface the cell envelope must be relatively large in comparison with the volume. Theoretically, therefore, the resistance of the target corpuscles should be increased, which corresponds with the observed facts.

The present investigation was not designed as a test of Haden's theory of fragility, and the evidence which it affords in favour of that theory by no means amounts to proof. Certainly many other factors besides osmotic distention must influence the hæmolysis of red blood corpuscles (Mason and Rockwood, 1924-25, Wolls, 1925). Nevertheless in the particular case of hæmolysis due to hypotonic sodium chloride solution it still seems possible that osmotic distention may normally be the determining factor and that the resistance of a corpuscle may depend upon the ratio of the area of the cell envelope to the total volume of the cell, but until it is possible to measure the surface and volume of erythrocytes without making assumptions concerning their shape it will be difficult to test the accuracy of this view.

#### SUMMARY

1. A special morphological type of red corpuscle is described which is constantly associated with the presence in the blood of an increased proportion of corpuscles resistant to 0.3 per cent

sodium chloride solution Evidence is given that the corpuscles of this type themselves possess increased resistance to hypotonic saline

2. In stained dry films this type of corpuscle has a characteristic appearance to which I have applied the term "target corpuscle" in wet films, however, it is bowl-shaped In formaldehyde solution the target appearance is assumed, and it was thus possible to observe the actual shape of the target corpuscle The correctness of the conclusions regarding the actual shape of the cells in dry and wet films is shown by X-ray photographs of plasticine models

3 Evidence is given that in blood containing cells of this type the red corpuscles are abnormally thin

4 The relationship between the morphology of erythrocytes and their resistance to hypotonic salt solution is discussed

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## INFLUENCE OF ASCORBIC ACID ON THE GROWTH AND TOXIN PRODUCTION OF *CL. TETANI* AND ON THE DETOXICATION OF TETANUS TOXIN

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RECENT studies in this department have shown that ascorbic acid (vitamin C) acts as a detoxicant of diphtheria toxin (Kligler, 1936, Kligler, Leibowitz and Berman, 1937) and of certain chemical poisons (KCN, phenol, Leibowitz and Guggenheim, unpublished observations), while its high reducing potency has enabled us to grow anaerobic bacteria (*Cl welchii*) under aerobic conditions (Kligler and Guggenheim, 1938). In the present paper we present the results of experiments on the influence of ascorbic acid on the growth and toxin production of *Cl tetani*. The procedure with regard to growth was essentially the same as that used with *Cl welchii*, while that relating to detoxication followed closely the technique used in the experiments with *C diphtheriae*. The experiments were conducted chiefly with a type II strain received from the National Collection of Type cultures. Control experiments were also made with strains of types I and III with the same results.

### *Effect of ascorbic acid on the growth of Cl tetani in the presence of air*

These experiments were made in meat extract broth. Just before inoculation, various amounts of ascorbic acid (Cebion, Merck) were added to give the concentrations shown in table I. Each tube was inoculated with the same amount of material from the supernatant fluid of a two-day culture grown in meat broth. The tubes were closed with cotton wool plugs and incubated at 37° C.

It will be noted (table I) that 0.1 part per thousand is not sufficient to produce conditions favourable for growth. On the other hand uniformly good growth occurred in tubes containing 0.25 parts per thousand or over. In this respect the results correspond with those obtained with *Cl welchii*. Addition of ascorbic acid above the minimum required does not further improve growth.

TABLE I.

Effect of percentage of peptone on the growth of *Cl. tetani* under aerobic conditions.

Percentage of peptone	Growth (turbidity at 37°C.)
0	4
1.0	—
0.5	4
0.25	4
0.1	—
0.0	—

In our experiments with *Cl. welchii* we noted that glucose had no effect on the minimum amount of vitamin required while peptone did but in cultures containing the minimum amount of vitamin the addition of glucose increased the density of growth. Similar experiments were carried out with *Cl. botuli*. Glucose exerted no influence either on the minimum of vitamin required for growth or on the intensity of growth. In this latter respect it differs from *Cl. welchii* but this difference is due to the fact that the tetanus bacillus does not ferment glucose. Peptone, however, exerted the same double effect noted with *Cl. welchii*, provided beef extract broth was used as a base. The results of a typical experiment are shown in table II. In general, a minimal concentration of 0.25 per cent. peptone is required for growth. Increase of peptone above that minimum both improves the density of growth and decreases the minimal amount of vitamin essential for the initiation of growth under aerobic conditions. A concentration of vitamin above the minimum does not influence the character of the growth.

TABLE II.

Effect of percentage of peptone on the minimal amount of vitamin required for aerobic growth of *Cl. tetani*.

Concentration of vitamin (I.U./ml.)	Percentage of peptone						
	0	0.25	0.5	1.0	1.5	2.0	3.0
1.0	—	—	—	—	—	—	—
0.2	—	—	—	—	—	—	—
0.05	—	—	—	—	—	—	—
0.01	—	—	—	—	—	—	—
0.0	—	—	—	—	—	—	—

In the experiment reported previously a double function was observed for the vitamin. It was essential for the aerobic growth of the organism and also for the production of gas.



natant fluid was tested on mice of approximately 15 g weight. These were injected subcutaneously in the right inguinal region with amounts of a given dilution equivalent to 0.25 c.c. per 15 g weight and were examined daily for 5 days. As far as possible we attempted to follow a constant and uniform procedure. The same strain varied from time to time in the potency of the toxin produced. Consequently each experiment had its own set of controls. On the whole the results were uniform and the differences between the toxicity of cultures with and without ascorbic acid clear cut and significant.

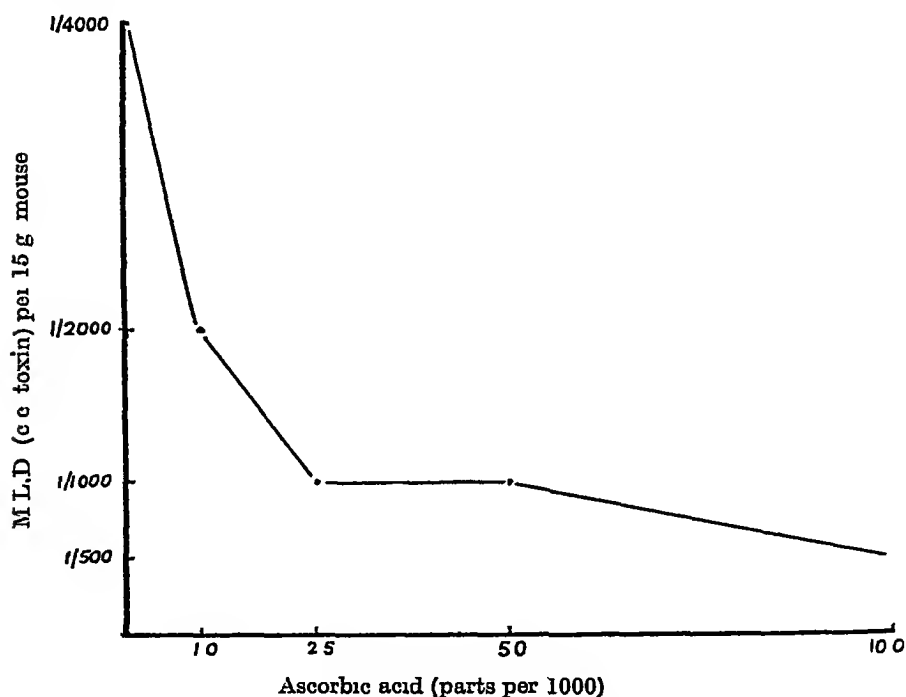


FIG. 1.—Inhibition of toxin formation in cultures of *Cl. tetani* in the presence of ascorbic acid (incubation for 4 days at 37° C.)

A summary of a number of experiments with 2.5 parts of vitamin per thousand parts of medium is given in table III. The table brings out the variability in the toxicity of different cultures, but it shows clearly that the vitamin-containing cultures were only one-fourth to one-tenth as toxic as the corresponding controls.

Further experiments showed that the degree of detoxication varies with the concentration of the vitamin. The results of a typical experiment are shown graphically in fig. 1. It will be noted that the toxicity of the cultures decreases with an increase in the concentration of ascorbic acid.

*Action of ascorbic acid on toxin*

In view of the effect of the vitamin on toxin formation in growing cultures we tested its effect on toxin as such. Four-day cultures were centrifuged and filtered through a Sartz filter. The toxin was kept in the ice box at about 5°C. The M.L.D. was  $\frac{1}{16,000}$  cc per 15 g and did not alter appreciably during storage at 5°C. The detoxicating effect of the vitamin was quantitated and striking. The results of two different experiments are shown in figs. 2 and 3 respectively. In fig. 2 is shown the effect of two different concentrations of vitamin after various time intervals and in fig. 3 the effect produced by various concentrations of the vitamin in 18 hours. It is evident that the degree of detoxication depends both on the time and on the concentration of the vitamin. Untreated toxin incubated under the same conditions did not decrease in potency during the period of incubation.

Incubation at temperature on the rate of inactivation is very marked. Tubes containing 50 parts per thousand ascorbic acid and appropriate controls were placed at 0.5°C and a similar set at 37°C. After an interval of 18 hours the toxicity was tested on mice. The M.L.D. of the toxin kept at the low temperature was  $\frac{1}{17,000}$  cc; the same is for the control. At 37°C the M.L.D. of the control was also  $\frac{1}{17,000}$  cc while that of the mixture with ascorbic acid was  $\frac{1}{10,000}$  cc.

The rate of detoxication was tested in still another way. In all the preceding experiments the vitamin was added to the concentrated toxin and the toxicity determined afterwards by diluting the fluid before injection into mice. We changed the procedure by first diluting the toxin so that 1 D.C. contained only 8 lethal doses. This diluted toxin was then divided among a series of tubes and varying concentrations of ascorbic acid added. The amount of vitamin per unit of toxin was therefore larger than in the previous experiments and consequently it was expected that more prompt neutralisation would occur. The toxin-vitamin mixtures were incubated at 37°C and tested after 3 and 24 hours respectively. 0.25 cc being injected subcutaneously. The results are summarised in table 13.

The detoxicating effect of the vitamin is clearly demonstrated by this experiment. Given a proper relationship between the concentrations of toxin and vitamin the detoxication is already

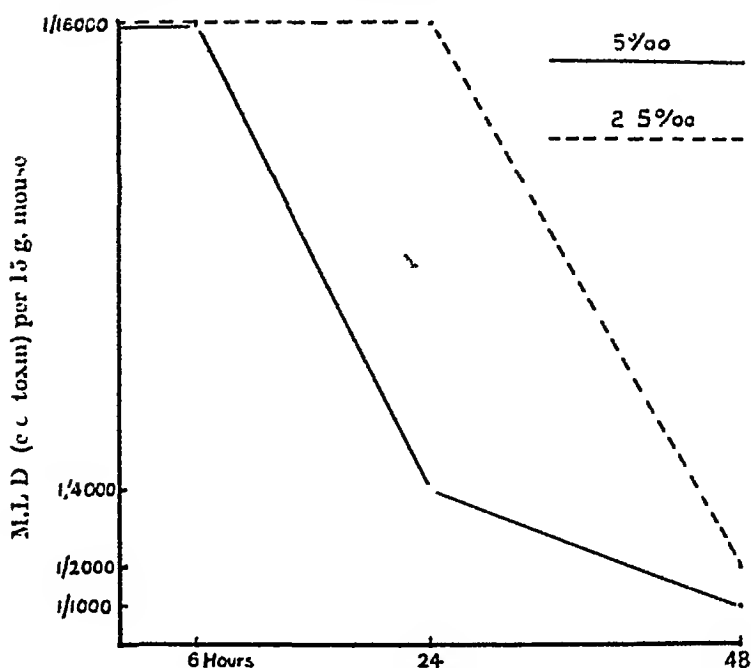


FIG. 2.—Course of detoxication of tetanus toxin by ascorbic acid at 37° C

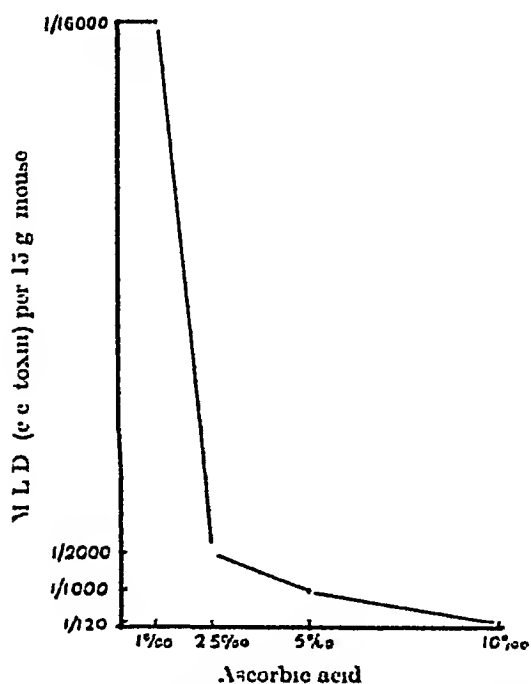


FIG. 3.—Effect of different quantities of ascorbic acid on the detoxication of tetanus toxin. (Time, 18 hours; temperature, 37° C)



evident after incubation for 3 hours at 37° C. In this respect the results correspond with those obtained with viruses (Kligler and Bernkopf, 1937) and chemical poisons (Leibowitz and Guggenheim, unpublished observations).

TABLE IV

*Detoxicating effect of various concentrations of ascorbic acid on diluted toxin*

Ascorbic acid (mg. per c.c. of fluid containing 8 M I D of toxin)	Result of injection of mixture incubated for	
	3 hours	48 hours
0.0	D	D
0.25	D	++S
1.0	++S	O
2.5	++S	O
5.0	++S	O

Each mouse received 0.25 c.c. subcutaneously or 2 M I D

D = died in 3 to 5 days

S = survived

++ = paralysis of leg

+ = mild paralysis of leg

O = no visible injury

#### *Fate of ascorbic acid in toxin vitamin mixtures*

In order to obtain further light on the nature of the interaction between vitamin and toxin, we examined in each experiment the rate of disappearance of ascorbic acid in toxin broth in relation to its spontaneous disappearance in broth (figs. 1-6). In fig. 1 are given results of experiments with two concentrations of vitamin (2.5 and 5.0 mg. per c.c.). It is noteworthy that the loss of vitamin in toxin broth and ordinary broth follows a parallel course, but is always greater in the toxin broth. Figs. 5 and 6 give the results of experiments on relative loss when different concentrations of vitamin are used. The differences between rate of disappearance of vitamin in toxin and ordinary broth are more striking the larger the concentration of vitamin. Fig. 5 gives the amount of vitamin found after 48 hours at 37° C., and fig. 6 the differences between the amount lost in toxin and in plain broth, also after 48 hours at 37° C. It should be noted that the degree of detoxication follows the same general trend, in other words that there is a relation between the amount of vitamin lost and the degree of detoxication.

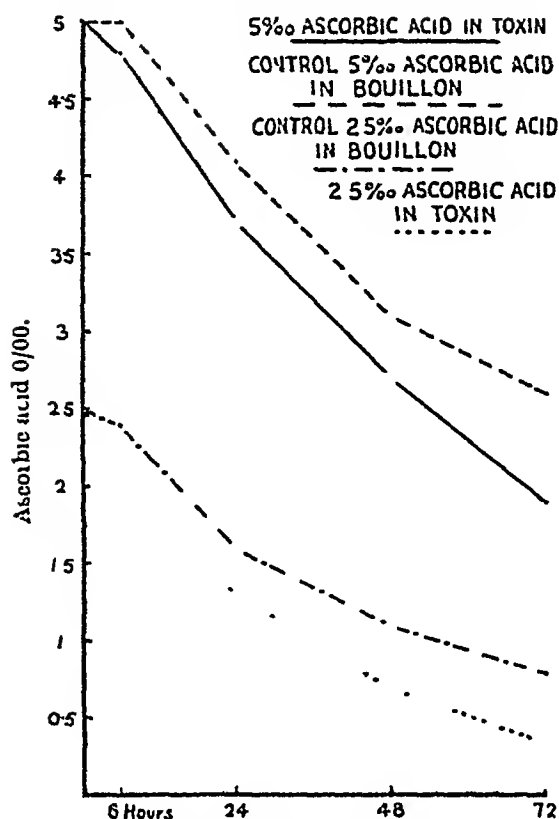


FIG. 1.—Disappearance of ascorbic acid in toxin and in ordinary broth.

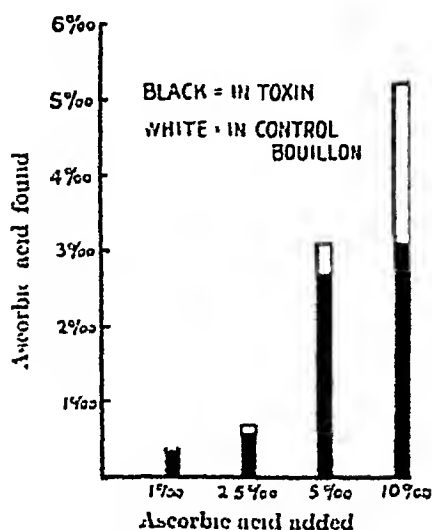


FIG. 2.—Disappearance of ascorbic acid in toxin broth and ordinary broth after 18 hours at 37° C.

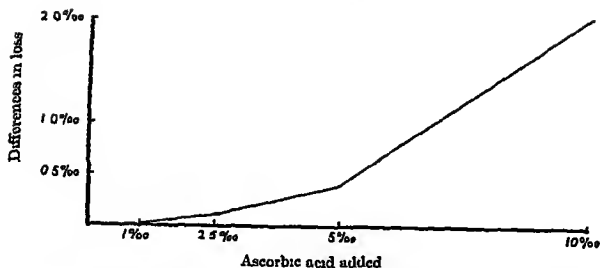


FIG. 6.—Differences in loss of ascorbic acid in toxin and in ordinary broth containing various concentrations of ascorbic acid (48 hours at 37° C.)

### DISCUSSION

The studies on the effect of ascorbic acid on diphtheria toxin and on viruses have raised the question as to the nature of the interaction. The results showed that there was a more rapid loss of ascorbic acid as a result of the reaction with toxin than in corresponding control fluids. Pursuing these studies in the chemical field in this laboratory, Leibowitz and Guggenheim (unpublished observations) have been able to show that this vitamin also detoxicates KCN and phenol and, by cryoscopic methods, that the ascorbic acid actually combines with these substances.

The experiments reported above carry these studies a step further. We have shown that ascorbic acid detoxicates tetanus toxin in much the same way as it does diphtheria toxin. The parallelism between the action of the vitamin on the two types of toxin is very close. In both cases it acts in the growing culture during toxin formation as well as on filtered toxin and the degree of neutralisation is a function of time and of the concentration of vitamin. In the case of tetanus toxin we have also found that the reaction is affected by temperature.

It should be noted that Schulze and Hecht (1937) have recently reported data suggesting a weakening of tetanus toxin by ascorbic acid, although their results were not definite because of the short incubation period used (90 minutes). Of interest also is the report by Harde and Benjamin (1934-35) that there was a reduction in ascorbic acid in the adrenals of guinea-pig treated with tetanus toxin—a finding analogous to our own in the case of diphtheria toxin.

It seems, then, that detoxication is a general property of ascorbic acid and that this role is performed in the body in the same manner as *in vitro*. What is the nature of this interaction? All experiments show that simultaneously with the detoxication

of the toxin or chemical poison there is a loss of vitamin which is in a general way proportional to the degree of detoxication and always more rapid than the spontaneous oxidative loss in corresponding control solutions

That oxidation is an essential part of the reaction is proved by the following experiment Two sets of tubes containing toxin and vitamin were prepared In one of the sets the air was removed by suction and the residual  $O_2$  absorbed by pyrogallie acid and sodium hydroxide Both sets of tubes, as well as the toxin controls (without vitamin), were incubated for 48 hours at  $37^\circ C$  and appropriate dilutions injected into mice. The results (table V) show that in the  $O_2$ -free tubes there is neither loss of ascorbic acid nor reduction in the potency of the tetanus toxin Oxygen and the oxidation of ascorbic acid are, therefore, essential to the detoxication of the toxin

TABLE V.

*Effect of ascorbic acid on tetanus toxin in the presence and in the absence of oxygen*

Content of tube	M L D (c c per 15 g)		Loss of ascorbic acid			
	In presence of air	Without air	With air		Without air	
			Amount (mg per c c)	Per cent	Amount (mg per c c)	Per cent
Toxin	$\frac{1}{8,000}$	$\frac{1}{8,000}$	.			
Toxin + ascorbic acid (2 5 parts per thousand)	$\frac{1}{1,000}$	$\frac{1}{8,000}$	1.75	70 0	0	0
Toxin + ascorbic acid (5 parts per thousand)	$\frac{1}{500}$	$\frac{1}{8,000}$	2 5	50 0	0 1	2 0
Broth + ascorbic acid (2 5 parts per thousand)		.	1 25	50 0	0	0
Broth + ascorbic acid (5 parts per thousand)			2 0	40 0	0 1	2 0

Our assumption is that the ascorbic acid actually combines with the toxin or poison and that the oxidation of this compound proceeds at a more rapid rate than that of ascorbic acid alone. This assumption harmonises with the facts thus far adduced The significant point, however, is that ascorbic acid acts as a general detoxicant The assumption that it serves that function also in the body explains the rapid and often extreme loss of this vitamin in guinea-pigs treated with diphtheria toxin and, as Harde and Benjamin have shown, also with tetanus toxin It may well be that this vitamin also performs this function with regard to toxic metabolic by-products, as is indicated by its action on phenol.

### Summary

It is possible to grow *Cl tetani* under aerobic conditions in broth containing a small amount of ascorbic acid. The amount of ascorbic acid required varies, within limits, according to the concentration of peptone. Even if large amounts of peptone are added no growth occurs unless a minimum of 0.05 parts per thousand of ascorbic acid are added.

Ascorbic acid added to growing cultures of *Cl tetani* reduces their toxicity in proportion to the amount added.

Ascorbic acid added to filtered tetanus toxin causes detoxication, the degree of detoxication varying with the temperature, concentration of vitamin and time.

Ascorbic acid disappears more rapidly in toxin broth than in ordinary broth.

On the basis of work in our laboratory with KCN, phenol, toxins and viruses it is suggested that detoxication is a major function of ascorbic acid and that it is brought about first by a direct combination of the vitamin with the toxin, virus or poison, followed by the oxidation of the new compound which destroys both toxin and vitamin. Detoxication in the test-tube occurs only in the presence of air.

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## SHORT ARTICLES

616 36—002 14 599 32

### THE INCIDENCE OF LEPTOSPIRAL INFECTION IN RATS IN LIVERPOOL

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With the object of determining the quantity and distribution of leptospiral infection present in Liverpool an examination has been made of 250 adult rats (*Rattus norvegicus*) caught in different parts of the city. The method of examination was to remove the rat's kidneys as aseptically as possible, decapsulate them and grind them up with about 20 g of sterile sand and 10 c.c. of sterile normal saline and examine the resulting emulsion for leptospiræ by dark ground illumination. It was found that the organisms may be seen much more easily if the tissue emulsion is first centrifuged at 2500 r.p.m. for 5 minutes. This deposits the larger particles leaving the leptospiræ and smaller particles in the supernatant fluid. When leptospiræ were seen 2 c.c. of the emulsion were inoculated intraperitoneally into a young white guinea pig. Leptospiræ were seen microscopically in 83 suspensions (33.2 per cent) and 83 guinea pigs were inoculated. Of these 51 died between the 6th and the 14th day after inoculation all showing the typical post mortem picture of infection with *L. icterohæmorrhagica*. By making cultures from heart blood in a medium containing 0.1 per cent peptone 0.1 per cent NaCl and 10 per cent rabbit serum 36 strains were isolated. When examined serologically by Schuffner's (1934-35) technique these strains were found to be a serologically homogeneous group identical with the Wijnberg strain of *L. icterohæmorrhagica*.

#### *The survival of guinea pigs after inoculation with rat kidney emulsions containing leptospiræ*

In the series of 83 inoculations with material containing leptospiræ 51 animals died of Weil's disease and 32 survived. Other workers have had similar experience. Buchanan (1927) in 61 inoculations had 13 survivors and Langworthy and Moore (1927) observed 9 failures to infect in 22 experiments. Taylor and Goyle (1931) failed to infect several guinea pigs with material from undoubted cases of Weil's disease and suggested that only young guinea pigs should be used as older animals might occasionally have some immunity to infection with leptospiræ. In the present series only young animals have been used but the same phenomenon has been met with. The results might be explained in a number of ways and four possibilities have been considered.

- (a) The leptospiræ in the kidney emulsions were not *L. icterohæmorrhagica*.
- (b) The leptospiræ were *L. icterohæmorrhagica* but the dose given to the guinea pig was insufficient to produce infection.
- (c) Certain guinea-pigs are immune to infection with *L. icterohæmorrhagica*.
- (d) In the inoculum of 2 c.c. of kidney emulsion there were sufficient antibodies derived from the rat to protect the guinea pig from fatal infection.
- (e) It is known that saprophytic leptospiræ exist widely in nature and

are indistinguishable morphologically from *L. icterohæmorrhagæ*. These organisms, however, do not persist in the animal body but are soon destroyed. Furthermore the sera of all surviving guinea-pigs were tested for antibodies against a saprophytic strain of *L. biflexa* isolated from Liverpool tap water with completely negative results. This evidence is not conclusive as we have some facts which suggest that there are local serological varieties of *L. biflexa*. When tested against *L. icterohæmorrhagæ* 14 sera out of 32 agglutinated this organism to titres ranging from 1/30 to 1/300. This fact we regard as evidence that certain of the survivors had been inoculated with *L. icterohæmorrhagæ*, particularly as we have failed to find such antibodies in normal guinea-pigs.

(b) The fact that some of the animals develop immune bodies might suggest that the inoculum did not contain sufficient organisms to produce death, but only enough to cause a mild infection with recovery. A rough record was kept of the numbers of leptospiræ seen in the kidney emulsions and these and the results of the inoculations are shown in table I.

TABLE I

*Relation of numbers of leptospiræ in suspension to infectivity*

Number of leptospiræ in inoculum	Number of guinea-pigs inoculated	Results of inoculation		Percentage mortality
		Died	Survived	
Very numerous	3	3	0	100.0
Numerous	46	26	20	56.5
Few	13	8	5	61.6
Scanty	9	7	2	77.8
Very scanty	4	1	3	25.0
Non-motile organisms only	8	6	2	75.0

The three inocula which contained a great number of leptospiræ produced fatal results in each case. In the remaining 80 inoculations the percentage of deaths is not very regularly related to the number of organisms seen, and it does not seem feasible to explain the results on this basis unless there is considerable variation in virulence.

(c) As already stated Taylor and Goyle believed old guinea-pigs to have some immunity to infection with pathogenic leptospiræ. I have described a naturally occurring infection with *L. icterohæmorrhagæ* in a guinea-pig (Mason, 1937) and pointed out that immunity might be produced by a slight natural infection. I have since examined 56 mature guinea-pigs for the presence of immune bodies to *L. icterohæmorrhagæ* and have failed to find any. It is therefore unlikely that such a large proportion as 32 out of 83 guinea-pigs would be naturally immune.

(d) The presence of a protective substance in the emulsions would explain perfectly the survival of certain animals. Two c.c. is a large bulk of tissue emulsion compared with the number of leptospiræ therein contained. The quantity of immune body would naturally vary with the amount of tissue and blood in the emulsion and the titre of the serum. To demonstrate the presence of immune bodies two rat-kidney emulsions containing leptospiræ were rapidly filtered through Pasteur-Chamberland L 3 filters. The filtrates were found to agglutinate *L. icterohæmorrhagæ*. Two c.c. of each filtrate and 2 c.c. of saline were each mixed with 0.1 c.c. of a virulent culture of *L. icterohæmorrhagæ*. Each mixture was then inoculated intraperitoneally.



into a guinea pig. The animal which received the virulent culture in saline died on the 8th day with typical signs of Weil's disease, while the others remained well. It is clear that in those particular emulsions there were sufficient protective substances to protect guinea pigs against an infective dose of virulent leptospiræ.

It seems justifiable to conclude that the leptospiræ seen in suspensions were really *L. icterohæmorrhagias* and that the incidence of this infection in the Liverpool rats examined was at least 33·2 per cent, the negative animal experiments being due in large part to simultaneous inoculation of protective substances. The real incidence may well be higher, as no inoculations were made with suspensions in which leptospiræ could not be seen.

Considering only those cases in which the microscopic findings were confirmed by animal inoculation the distribution of the infected rats according to source is recorded in table II.

TABLE II  
Frequency of leptospiral infection in rats from different sources

Source	Number examined	Infected	
		Number	Per cent
Sewers	180	57	31·7
Private houses	39	6	15·4
Docks	22	2	9·0
Public places and gardens	9	0	0

The difference in incidence between house rats and sewer rats is not certainly significant ( $\frac{D}{SE} = \text{approximately } 2$ ) and all that these figures show is that the infection is not confined to sewer rats. The infected rats came from all parts of the city and showed no tendency to predominate in any particular locality.

#### Sub infections with *L. icterohæmorrhagias*

Alton and Brown (1933) examined the sera of 45 sewer workers who had no recollection of ever having been jaundiced. In 9 cases antibodies to *L. icterohæmorrhagias* were demonstrated to titres ranging from 1/30 to 1/100 and these sera were shown to protect guinea pigs from fatal infections with virulent *L. icterohæmorrhagias*. Davidson and Smith (1936) examined the sera of 210 fishworkers, of 17 giving a history of jaundice 15 were shown to have immune bodies to titres between 1/30 and 1/1000. In 36 of the remaining 193, immune bodies were demonstrated to titres between 1/30 and 1/300. In Liverpool only 3 cases of leptospiral infection have been diagnosed in the past 5 years but it appeared worth while to look for evidence of sub infection in a staff of rat catchers permanently employed in work where infection with *L. icterohæmorrhagias* should be frequent. The sera of 11 of these men, none of whom recollected having been jaundiced were examined by Schuffner's technique. Six sera agglutinated *L. icterohæmorrhagias* to titres varying from 1/30 to 1/300. Protection tests were also done in which guinea pigs were inoculated subcutaneously in the hind leg with 0·5 c.c. of serum from a rat catcher after which they were given 0·2 c.c. of a virulent culture of *L. icterohæmorrhagias* intraperitoneally. The five animals which received the sera in which no immune bodies had

been detected by the agglutination test all died between the 7th and 9th days after inoculation. Five animals receiving sera with an agglutination titre between 1/100 and 1/300 all survived. One guinea-pig receiving serum with a titre of 1/30 developed jaundice on the 10th day but recovered. It is therefore most probable that six of these eleven men had suffered a sub-infection with *L. icterohæmorrhagæ* with the production of immunity.

### Summary

1 In a series of 250 rats leptospiræ have been demonstrated microscopically in kidney suspensions in 83 (33.2 per cent) and by animal inoculation in 65 (26 per cent).

2 The survival of guinea-pigs after inoculation with material from rats seen to contain leptospiræ appears to be dependent on the presence of protective substances in the suspension.

3 Of 11 sera obtained from men employed as rat-catchers 6 contained antibodies to *L. icterohæmorrhagæ*.

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576.809.483 : 576.851.5 (*Bac. prodigiosus*)

### THE EFFECT OF VARIOUS MEAT EXTRACTS ON PIGMENT PRODUCTION BY *B. PRODIGIOSUS*

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In a previous communication to this Journal (Goldsworthy and Stall, 1936) attention was drawn to certain conditions conducive to pigment production by *B. prodigiosus* on solid media. These conditions were the absence of meat extract (ox heart) and glucose, the presence of one of several other carbohydrates and incubation at low temperatures. It was concluded that the use of mannitol peptone agar and incubation at 14-18° C afforded the most satisfactory conditions for pigment production.

In this note we record some subsequent observations on the effects of different meat extracts. The two strains of *B. prodigiosus* used in the earlier investigation were again used. These strains, after cultivation for several months under the conditions favourable to pigment production,

had been stored on cooked meat medium under liquid paraffin at 4° C for about 18 months

The media were prepared from simple peptone agar with and without the various meat extracts and were used either unaltered or with added glucose or mannitol. The experiments were carried out at 37° and 14 18° C and readings were made at intervals up to 5 days. The results are summarised in the table

TABLE

*Effect of various meat extracts on the production of pigment by B prodigiosus*

Extract	STRAIN V						STRAIN 1377					
	37° C (42 hrs)			14 18° C (66 hrs)			37° C (42 hrs)			14 18° C (66 hrs)		
	Nil	Glucose	Mannitol	Nil	Glucose	Mannitol	Nil	Glucose	Mannitol	Nil	Glucose	Mannitol
Ox heart	—	—	—	—	—	—	—	—	—	++	—	—
Beef	—	—	—	—	—	—	—	—	—	++	tr	—
Veal	—	—	—	—	—	—	—	—	tr	—	—	—
Horse meat	—	—	—	—	—	—	—	—	—	—	—	—
Lab Lemo	—	—	—	—	+	++	—	++	++	++	++	++
Nil	—	—	—	—	++	+++	—	+	+	++++	++++	+++

++++ = a colour as intense as that of a layer of blood 1 mm thick  
 — = absence of pigment tr = a very slight trace

At 14 18° C, the addition of extract of ox heart, beef, veal or horse flesh to mannitol peptone agar inhibited pigment production by strain V completely. With strain 1377 the inhibition was less and depended upon the nature of the extract. The inhibitory effect of these extracts was slightly more apparent at 37° C. When Lab Lemo was added to the media in place of meat extract, pigment production by strain 1377 was not inhibited at 14 18° C, either in the presence or in the absence of added carbohydrates. With strain V there was no inhibition in mannitol peptone agar and only slight inhibition in glucose agar. Thus Lab Lemo differs from the other meat extracts in that, in the concentration used it caused little or no inhibition of pigment production by *B prodigiosus* on the three peptone agar media tested.

It is also shown in the table that strain V did not produce pigment at 37° C, and strain 1377 produced little. This absence of pigment production at 37° C agrees with the published results of other workers and of ourselves. However, both organisms in the first subculture from the storage medium exhibited excellent pigment production at this temperature on certain of the media which did not contain extract of either ox heart, beef, veal or horse flesh.

The inhibitory effect of glucose previously reported was rather irregular in this series of experiments.

#### Summary

Extracts of ox heart, beef, veal and horse flesh inhibit the production of pigment by *B prodigiosus* on an otherwise very satisfactory solid medium. Lab Lemo has little or no such effect.

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GOLDSWORTHY, N. E., AND STILL, J. L. 1936 this Journal, xlii 555



## BOOKS RECEIVED

## The titration of 2-allyl cat at 1 atmosphere data

1. The first step is to identify the key components of the system. This involves understanding the hardware, software, and data involved. For example, in a web application, this might include the server, the database, and the user interface.

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 various sources of intelligence.

[illegible]

With the exception of a few obvious errors, the examples to be read for the student's use in the present volume should prove very useful and should be of value wherever I have assumed them in compiling the text. The only volume cited in Dr. Fiske's book - it is no person on his work for - is that of some probably no different investigators don't find it all easy to read or useful work. I repeat repetition of the text but if it is useful by the author but in a perusal of the book, etc., as to the impression that this has been carried rather far in the text of the novel in its use.

### The postmortem examination.

By SIDNEY FARBER Springfield, Ill., and Baltimore, Md.  
C. C. Thomas. 1937. Pp xii and 201, frontispiece and 32 text figs  
16s.

This book was written "in response to a need made evident in the teaching of medical students, young pathologists, and clinical house-officers who are able to spend but a short time in the laboratory before assuming ward duties" It may be commended as a very full and careful account not only of the actual method of examining the dead body but also of the equipment of the post-mortem room It is certainly a book for the pathologist but we doubt whether students, who have more than enough to read as it is, would not do better to stick to practical instruction so far as autopsy technique is concerned

The equipment of the post-mortem room is very completely detailed, even to the provision of "a suitable wall inscription, very often in Latin (for example, *Mortui vivos docent*)" An elaborate and obviously expensive post-mortem table is described, including a ventilating duct in the hollow base, connected with a fan in the roof of the building to carry off the odours Yet it is suggested (presumably as a measure of economy) that in the refrigerator where the bodies are stored the upper compartments may be used for the storage of culture media

To some of the procedures recommended strong objection must be offered. It is advised that the duodenum be opened in the midline, either *in situ* (p. 74) or after removal from the body along with the stomach, liver and pancreas (p. 125) An enterotome is then inserted through this opening and a midline longitudinal cut made in the anterior wall as far as the pylorus, after which the incision is continued along the greater curvature of the stomach Such a proceeding will almost certainly cut through an anterior-wall duodenal ulcer or scar if such be present (and duodenal scars are not always easily demonstrable, even if undamaged, nor are they always visible from the serosal aspect). The only way to open the *first part* of the duodenum is along the lesser curvature, *i.e.* in line with the greater curvature of the stomach. By this means nearly all ulcers and scars of the duodenum will be avoided. Only large ulcers of the posterior wall which have transgressed the lesser curvature of the duodenum run any risk of damage Here as elsewhere in the book, the impression is left that the author is more at home in the infantile than in the adult body

It is suggested (p. 76) that "A long flat beak incision" should be made through the middle of the liver from left to right, in order to facilitate handling during removal. It is advised (p. 43) that "The cut surfaces of the organs should not be washed with water, lest they be spoiled thereby for successful histologic preparations," yet they are to be cleaned "by gentle strokes of the knife edge", or "the surfaces . . . may be washed slightly with normal saline" Certainly in special instances the use of water may be objectionable, but for most organs it is perfectly feasible to take the tissue required for histology from some other part than the surface which has been washed Even washing with water, if it is only momentary, does little harm.

We cordially agree with the author that scrubbing brushes (for toilet purposes) should not be permitted in the post-mortem room, but to rinse the hands and arms in 70 per cent alcohol (after having washed them "with running water and liquid soap for five minutes") seems an unnecessary extravagance.

[illegible][illegible]

### Effect of Temperature on Tissue and Plasma

[illegible][illegible]

The first of these is the fact that the number of individuals in the population is not constant, but varies with the conditions of the environment. In the case of the bacteria, the number of individuals in the population is not constant, but varies with the conditions of the environment. In the case of the bacteria, the number of individuals in the population is not constant, but varies with the conditions of the environment.

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**A Festschrift to Sir John M'Fadyean**, being the jubilee number (vol L, part 1) of the *Journal of Comparative Pathology and Therapeutics*, December 1937.

Croydon H. R. Grubb, Ltd Pp. vi and 230, with numerous portraits. 7s. 6d

"When a man has . . . rendered what . . . is clearly perceived to be a signal service through his own efforts and initiative, to the lasting good of his fellow men, it would seem that nothing should deter giving expression on fit and proper occasion within his lifetime to the natural, human response of gratitude. Such occasion, it was felt, had arisen at the conclusion of the 50th volume of the *Journal of Comparative Pathology and Therapeutics*, which had been founded in 1888 as a private enterprise by Sir John M'Fadyean, and has since been edited by him almost entirely single-handed." On this occasion, the editor consented to the request of his colleagues that they be given a free hand in the production of the final number of the 50th volume and the result is the very remarkable tribute to a great pioneer which is now before us. There are appreciations of the man and his work from a large group of friends and admirers, including Sir Robert Muir, Sir John Ledingham, Professor Sharo Jones and Professor Wooldridge in this country, and Professors von Ostertag, Schmaltz, and Leclanche and Dr John R. Mohler abroad. A series of special articles deals with (1) the journal, (2) the man, (3) his disciples and (4) Sir John as a speaker in council and debate. Dr R. E. Glover of the National Institute for Medical Research contributes a survey of Sir John M'Fadyean's contributions to the study of tuberculosis, Mr W. A. Pool a note on his work on "Louping-ill" and Dr D. S. Rabaghiani an account of "Present-day administration of the milk and dairies legislation as influenced by the work and teachings of Sir John M'Fadyean." There are numerous original contributions on veterinary medicine and pathology, many of them from distant parts of the Empire. Selected passages from the writings of the master are interspersed and finally there is a full bibliography of Sir John's contributions to his own journal, and a list of his chief contributions to the *Journal of the Royal Agricultural Society of England*. A very attractive feature is the excellent series of portraits, including four of Sir John himself at various ages, and others of the contributors of the principal "appreciations."

A great man worthily honoured

**Medical bacteriology descriptive and applied, including elementary helminthology.**

By L. E. H. WINTER. Third edition. London: J. & A. Churchill, 1938. Pp. ix and 372; 79 text figs. 11s. 6d.

The previous edition of this book has already been reviewed in this *Journal* (1934, xxxix, 545). It is unnecessary, therefore, to recapitulate the main outline of the book and to indicate again the better and the less satisfactory features which have already been discussed. One or two of the errors pointed out in that review have been corrected. The statement that boiled blood agar can be used as a satisfactory substitute for Bordet-Gengou medium in the isolation of *B. pertussis* still stands. In this as in a number of other instances, the author leaves the responsibility of lack of personal experience of the subject discussed, if there is no attempt to describe the extremely characteristic colony of *B. pertussis*







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